University of Alberta

Characterization and analysis of two markers of macrophage development and function: Prominin and tumor necrosis factor-α of the goldfish (*Carassius auratus*)

by

John Gerard Walsh ©

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ABSTRACT

A competent inflammatory response requires both the continuous production of new effector cells such as macrophages, and cytokine regulation of their antimicrobial mechanisms. My research characterized prominin and examined the pro-inflammatory role of TNFα in goldfish. Goldfish prominin was highly expressed in the kidney and gills. Furthermore, prominin mRNA levels were high in the macrophage progenitor subpopulation, less in mature macrophages and the prominin mRNA was not expressed in monocytes. Two isoforms of goldfish TNFα (1 & 2) were cloned, sequenced and recombinant TNFα-2 generated using a prokaryotic expression system. TNFα-1 was constitutively expressed while TNFα-2 expression was associated with activated macrophages. Recombinant goldfish TNFα-2 was found to induce chemotaxis, phagocytosis and nitric oxide production in goldfish macrophages.

Goldfish prominin and TNFα were found to be excellent markers of goldfish macrophage development and activation, respectively. Characterization of these molecules enhances our ability to study the innate immune response of fishes.

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LIST OF ABBREVIATIONS

ANOVA-analysis of variance
BLAST-Basic Local Alignment Search Tool
BSA-bovine serum albumin
C-chemokine
CC-chemokine
CCM-cell-conditioned medium
cDNA-complementary DNA
CGD-chronic granulomatous disease
CK-1-chemokine-1
Con A-concanavalin A
CR-complement receptor
CXC-chemokine
CXCL-CXC ligand
CXXXC-chemokine
DNA-deoxyribonucleic acid
dsRNA-double stranded RNA
ECM-extracellular matrix
FACS-fluorescence activated cell sorter
GMCL-goldfish macrophage cell line
GM-CSF-granulocyte macrophage colony stimulating factor
HKL-head kidney leukocytes
ICAM-intercellular adhesion molecule
IFN-interferon
Ig-immunoglobulin
IL-interleukin
IL-1β-interleukin 1-β
IL-1R-IL1 receptor
IPTG-isopropyl-beta-D-thiogalactopyranoside
IRF-interferon regulatory factor

L-NAME- N-Nitro-L-Arginine Methyl Ester
LPS-lipopolysaccaride (bacterial endotoxin)
LT-β-lymphotoxin-β
MAF-macrophage activation factor (mitogen activated cell supernatants)
MHC-major histocompatibility complex
mRNA-messenger RNA
NCS-newborn calf serum
NADPH- nicotinamide adenine dinucleotide phosphate (reduced form)
NCBI-National Center for Biotechnology Information
(NF)-κB-nuclear factor-κB
N^G-MMLA-Monomethyl-L-arginine
NK-natural killer
NO-nitric oxide
NOS-nitric oxide synthase (i-inducable, e-endothelial, n-neuronal)
ORF-open reading frame
PBL-peripheral blood leukocytes
PCR-polymerase chain reaction
PDBu-phorbol dibutyrate
PDTC-pyrrolidine dithiocarbamate
PHA-phytohemagglutinin
Phox-phagocyte oxidase
PKM-primary kidney macrophages
PMA-phorbol 12-myristate 13-acetate
Poly I:C-polycytidylic acid
PRR-pathogen recognition receptors
PTX-pentoxifylline
RACE-rapid amplification of cDNA ends
ROS-reactive oxygen species
rTNFα-recombinant TNFα
RT-PCR-reverse transcriptase-polymerase chain reaction
TGFβ-transforming growth factor-β

TNFα-tumor necrosis factor alpha

TNFR-TNF receptors

CHAPTER 1

GENERAL INTRODUTION AND OBJECTIVES

1.1 Introduction

The importance of the immunological memory, the hallmark of an adaptive immunity, is often highlighted as the key component of host defense against pathogens. The fact is that the majority of organisms on this planet do not have an adaptive immune response and therefore must rely exclusively on innate immune mechanisms to defend against pathogens. Clearly this has not limited the diversity and prevalence of the majority of metazoans suggesting innate immunity is of central importance for their existence. The bony fishes (teleosts) possess an adaptive immune system, but one that is extremely limited in comparison to that of mammals and therefore bony fish must presumably rely heavily on the innate immune response. Despite this, they are arguably the most successful of all vertebrate groups. An investigation of the immune responses of the teleosts is therefore important in order to understand the evolution of immune mechanisms in vertebrates.

Leukocytes including macrophages are central cells of the innate immune response. One of the integral components of innate immunity is the process called inflammation. Macrophages participate in the inflammatory responses of vertebrates at various levels: upon activation they have the ability to locate at the inflammatory sites through the process known as chemotaxis and upon arrival to these sites they exhibit potent antimicrobial responses [1-2].

A competent inflammatory response requires the continuous production of new effector cells. The source of these new cells is the hematopoietic progenitor populations located in the bone marrow of mammals or the kidney of fish. In mammals, members of the prominin family are markers of hematopoietic progenitor populations [3] and the characterization of this molecule in goldfish will allow for the identification of hematopoietic pathways that are of critical importance for the induction of fish inflammatory responses. Another important marker of a competent inflammatory response is tumor necrosis factor alpha (TNFα), a pleiotropic pro-inflammatory cytokine produced by macrophages [4]. The characterization of this cytokine in goldfish is critical for understanding host defense against pathogens because this molecule has a central regulatory role in inflammation and innate immunity.

1.2 Objectives of the Thesis

The main objective of my thesis was to examine the pro-inflammatory role of TNFα in the goldfish. My second objective was to characterize a novel hematopoietic molecule of the goldfish. The specific aims of my thesis work were:

1) To examine the expression of goldfish TNFα with particular emphasis on the expression of two TNFα isoforms (TNFα-1 and 2) in cultured goldfish macrophages. Expression was investigated for various states of development and activation.
2) To produce a recombinant goldfish TNFα protein using a prokaryotic expression system and to test for biological activity of the recombinant molecule. Chemotaxis, phagocytosis and the production of nitric oxide were examined.
3) To fully sequence a prominin molecule from goldfish and to characterize its expression in both whole tissues and macrophage developmental stages.

1.3 Outline of Thesis

In the second chapter of this thesis I review the role of fish phagocytes in the inflammatory response with specific emphasis on adhesion, chemotaxis, phagocytosis, the production of antimicrobial compounds and the role of pro-inflammatory cytokines. Chapter 3 is a comprehensive description of the materials and methods used over the course of this work. In chapter 4, I describe the characterization of goldfish tumor necrosis factor-α (TNFα) both it terms of its mRNA expression and its functional effects on goldfish macrophages. Specifically two isoforms of goldfish TNFα (1 and 2) were cloned and sequenced. A recombinant TNFα-2 molecule was produced using a prokaryotic expression system. Using the goldfish TNFα-2 recombinant I demonstrated a role for goldfish TNFα in the regulation of chemotaxis, phagocytosis, nitric oxide production as well as cytokine expression in teleosts. In the fifth chapter I discuss the identification and characterization of a novel heamatopoetic progenitor cell marker known as prominin in the goldfish. The open reading frame of goldfish prominin was fully sequenced and its expression in whole tissues as well as *in vitro* cultured macrophage subpopulations was examined using real-time PCR techniques. Goldfish prominin was highly expressed in the kidney and gills, and at lower levels in other tissues examined. Macrophage progenitor populations also highly expressed prominin with decreased expression observed in mature macrophages and monocytes. Chapter six is a general discussion of the importance of prominin and tumor necrosis factor-α as macrophage markers of activation and development, the two processes of central importance for competent inflammatory and antimicrobial responses.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Inflammatory responses can be initiated by factors released from dead/damaged cells (danger theory) or by the recognition of microbial signatures that trigger the response (pattern recognition theory). As the inflammatory response develops, both internally derived signals (i.e. cytokines) and microbial-patterns (i.e. antigen presentation) continue to communicate information to a coordinated immune system [1]. Thus, the recruitment of phagocytic leukocytes as part of the early inflammatory response could be viewed as a major point of convergence that makes sense of a seemingly chaotic milieu of signals.

In fish the existence of macrophages and neutrophils has been well established. Also, the innate immune system of fish is of particular interest in light of the perceived deficiencies in their adaptive immune response. In this chapter, the involvement of fish phagocytes in the inflammatory response was reviewed with specific emphasis on adhesion, chemotaxis, phagocytosis, the production of antimicrobial compounds and the role of pro-inflammatory cytokines.

2.2 Adhesion

While resident tissue phagocytes (primarily macrophages) are present at the first moments of an emerging challenge by pathogens, the vast majority of the phagocytes involved in the inflammatory response must be recruited to the lesion site from the bone marrow and circulating populations passing through the local vasculature. After an inflammatory stimulus, endothelial cells up-regulate their presentation of adhesion molecules including the selectins and the immunoglobulin super family intercellular adhesion molecules (ICAMs). In mammals, the selectins bind to specific oligosaccharide ligands on the surface of phagocytes, while ICAMs interact with integrin molecules on the surface of the leukocytes [2].

The next step in the recruitment of leukocytes to the inflammatory lesion site is called diapedesis, and it involves the translocation of the leukocyte across the endothelial barrier through temporary alteration of the gap junctions. This process is rapid and involves significant rearrangements of the leukocyte cytoskeleton [5].

To date, no endothelially expressed adhesion molecules have been cloned from fish. Despite this, there is evidence that adhesion molecules on the endothelial surface are up-regulated upon stimulation by activating factors. Matsuyama and Iida [6] found that pre-treating Tilapia endothelial cells with the products of degranulating mast cells enhanced neutrophil adhesion to the endothelial surface 6 fold over untreated cells after 6hrs. Adhesion was also enhanced by pre-treatment of the cells with neutrophil lysates as well as bacterial endotoxin (lipopolysaccaride or LPS) or zymosan treated sera [6]. Morphologically, the adherent neutrophils took on a polarized or mirror shape, suggesting their readiness to migrate across the endothelial surface [6].

2.2.1 Integrins

Expressed on the surface of a variety of cell types including leukocytes are a group of molecules known as integrins. The integrins are a family of $\alpha\beta$ heterodimeric receptors that are essential for adhesion interactions with other cells as well as with the extra-cellular matrix. On the extracellular matrix (ECM) they interact with components such as collagen, laminin and fibronectin. On the cell surface they interact primarily with ICAMs. Integrins have been identified across the animal kingdom and significant diversity of integrins exists in the bony fishes. For example, a group of α integrins referred to as leukocyte-specific (I-domain containing subunits) are absent from pufferfish although they have been identified in carp and zebrafish [7].

Prior to the identification of specific adhesion molecules in fish, research suggested conserved mechanisms were underlying fish leukocyte adhesion. In the gilthead seabream, a monoclonal antibody raised against an unidentified leukocyte surface molecule was found to induce aggregation when added to cultured neutrophils [8]. The aggregation response required the presence of Ca^{2+} and was blocked by the cytoskeletal inhibitor cytochalasin B. These findings suggested a role for intracellular Ca^{2+} signaling and the active rearrangement of cytoskeletal elements. This surface adhesion molecule was also responsive to activating signals secreted from stimulated leukocytes as well as bacterial endotoxin (LPS) [8]. In catfish, neutrophil adhesion to the extracellular matrix (ECM) protein fibrinogen was also found to be inhibited by the chelation of Ca^{2+}. Furthermore, the addition of Arginine-Glycine-Aspartic Acid (RGD) tri-peptides, that represent the integrin binding site on many ECM proteins, resulted in an 86% reduction in the binding of neutrophils to fibrinogen [9].

β2 integrins are molecules responsible for the tight adhesion of neutrophils and macrophages and their identification in fish was an important discovery with regard to leukocyte adhesion. A β2 integrin on the surface of catfish neutrophils was up-regulated by stimulation with phorbol dibutyrate (PDBu) an activator of protein kinase C which was implicated in the regulation of adhesion molecule expression [10]. In rainbow trout, stimulation of a monocyte cell line (RTS11-N) with phorbol 12-myristate 13-acetate (PMA) enhanced adhesion to denatured bovine serum albumin (BSA). This stimulation and increased adhesion coincided with an up regulation of CD18 (β2 integrin) [11]. Although other molecules could have been responsible for the direct interaction, the adhesion enhancing capability of $MnCl_2$ also pointed toward integrin involvement. The stimulated cells took on a spreading morphology similar to a related macrophage cell line RTS11-A that was typically adherent even in the absence of stimulation [11].

Novel β integrins have also been identified on fish leukocytes [12-14]. For example, catfish β1 integrin was found to be present on the surface of peripheral blood leukocytes (PBL) as well as neutrophils. The catfish β1 integrin was similar to mammalian β1 integrins as well as those from other non-mammalian vertebrates including chicken and *Xenopus* [12]. β integrins have also been identified in carp and trout [13][14].

2.3 Chemotaxsis

Leukocytes adhering to vascular epithelium as well as ECM proteins near the inflammatory site do so after recognizing and responding to activating and attracting compounds many of which function to induce a strong chemotactic response. It may be expected that any ligand released by pathogens, or in response to pathogens, regardless of its primary role may generate a chemotactic gradient and be recognized by the cells possessing the receptor for that ligand. However, certain substances namely the chemokines and anaphylatoxins such as C3a and C5a, are known to induce strong leukocyte chemotactic response [15-16].

2.3.1 Chemokines

Chemokines are a superfamily of small peptides and in the context of inflammation are key modulators of chemotaxis and adhesion [15]. There are four chemokine subgroups based on the positioning of conserved cysteine residues; CXC, CC, C and CXXXC. Molecules belonging to the CXC and the CC subgroups have been most extensively studied in fish [15].

Of the members of the CXC subgroup that have been found in fish, several are related to the IL-8 or CXCL-8 molecules of mammals. The first chemokine identified was a CXCL-8 from the lamprey [17]. This chemokine was also identified in banded dogfish and silver chimaera [18-19] as well as the Japanese flounder and trout [20-21]. CXCL-8 was shown to be up-regulated in tissues and leukocytes upon stimulation with compounds such as LPS [20-21]. Despite this shared identity with mammalian CXCL-8, all fish CXCL-8s lack the ELR motif thought to be required for the chemoattraction of neutrophils [15]. Trout CXCL-8 does have a similar DLR motif [21].

Other CXC chemokines that have been identified in fish include several from catfish that share similarities with CXCL-10, 12 and 14 [22-23]. Little is known about the function of these molecules, although it has been speculated that CXCL-10 may have more of a role in the regulation of lymphocyte responses [22], while CXCL-12 and14 may play a role in fish development [23]. Two novel CXC chemokines as well as CXC receptors 1 and 2 were recently identified carp and were found to be up-regulated in kidney leukocytes in response to PMA stimulation but not LPS [24].

Representatives from the CC subgroup of chemokines identified in fish include those from trout, carp and Japanese flounder [25-28]. A recombinant trout CC chemokine CK-1 has been shown to induce chemotaxis in peripheral blood leukocytes (PBL) [29]. PBL mRNA isolated from fish that received inter-peritoneal injection of phytohemagglutinin (PHA) showed a large enhancement of CK-1 expression, unlike the trout macrophage cell line RTS11 which did not express CK-1 even after stimulation with PHA [29]. It has also been reported that PBL isolated from Japanese flounder that had been previously injected with CC chemokine cDNA, have enhanced chemotactic and super oxide responses [28].

2.3.2 Anaphylatoxins

Anaplylatoxins play a central role in chemoattraction of immune cells to inflammatory sites. These molecules are produced by the cleavage of the complement factors C3 and C5 and are referred to as anaphylatoxin C3a and anaphylatoxin C5a. The complement system is highly conserved in metazoans and functional anaphylatoxins have been identified in most fishes. The chemotactic response of hagfish leukocytes was greatly enhanced by purified mammalian anaphylatoxin C5a [30]. Studies of bony fish such as carp and trout found C5a to be highly chemoattractive for PBLs, head kidney leukocytes (HKL) as well as isolated neutrophils [31-33]. The des-arginase form of the C5a molecule which was inactive in mammals was also ten fold less chemoattractive to trout cells [34]. These data suggest that the mechanisms for the induction of the chemotactic response by C5a might be similar between mammals and fish. Using a fluorescently tagged trout recombinant C5a, Holland and Lambris [34] identified a C5a receptor on the surface of HKLs as well as PBLs. The binding to this receptor was significantly reduced by pre-incubation with non-fluorescent trout C5a but not with mammalian C5a indicating a greater specificity in the homologous system. The cells responding to trout C5a were primarily neutrophils [34]. C3a isoforms that have been tested in fish were found to be significantly less potent for induction of chemotactic response [33-35].

2.4 Phagocytosis

Upon reaching the site of infection both macrophages and neutrophils alter the inflammatory site by enhancing the antimicrobial response. Their primary role is containment and elimination of the microorganisms from the inflammatory site through the process of phagocytosis. Phagocytosis is often enhanced through the interaction of receptors expressed by phagocytes (FcRs and complement receptors) with serum components (immunoglobulin, complement proteins) that form an opsonizing coat on the surface of invading pathogens. Direct ligand receptor interactions between the microbe surface molecules and receptors such as integrins can also significantly enhance the phagocytic response [36].

2.4.1 Antibody-independent phagocytosis

As was mentioned previously, the highly conserved complement system is highly developed in fish. A role for complement cleavage products (anaphylatoxins) in the chemotactic response has already been discussed. There is also an important role for anaphylatoxins in the activation of inflammatory phagocytes [32]. However, in terms of complement opsonization, it is the C3b and C4b complement proteins that play the key role during inflammation [32].

Even in the absence of specific reagents (i.e. recombinant fish C3b) it is possible to control for the activity of complement because these molecules are heat labile as well as sensitive to the chelation of certain cations such as Ca^{2+} and Mg^{2+}. In catfish complement was found to be important in the attachment of PBLs to bacteria as well as latex beads. This opsonization effect was inhibited by heat inactivation of the serum, EDTA treatment and C3b adsorption [37]. In addition, coating of agarose beads with human C3b enhanced salmon macrophage attachment to, and phagocytosis of these same beads [38].

2.4.2 Antibody-dependent phagocytosis

There is also evidence for the presence of a functional antibody response and antibody-phagocyte interactions in both cartilaginous and bony fishes [39-42]. In salmon, the phagocytosis of *Aeromonas salmonicida* by macrophages and neutrophils was not only enhanced in the presence of immune serum but was also dependent on the presence of active complement [40]. Independent of complement, IgM-mediated opsonization and phagocytosis was reported for nurse shark neutrophils [39]. This response was blocked by the addition of an anti-shark IgM monoclonal antibody or by the microtubule inhibitor cytochalasin [39]. Antibody-dependent opsonization and phagocytosis was also observed in Atlantic salmon and was independent of complement [41]. Antibodies were shown to mediate the phagocytosis by trout leukocytes of the bacterium *Yersinia ruckeri* [42]. Furthermore, carp macrophages where found to bind and internalize antibody, and following internalization the binding capacity was regenerated on the macrophage surface [43].

2.4.3 Complement/immunoglobulin receptors and phagocytosis

To date no CR1 or CR3 complement receptor homologs have been identified in fish. However, an unidentified receptor for complement on the surface of trout erythrocytes has provided corroborating evidence for the existence of a CR1 receptor in fish. Early experiments in the nurse shark used affinity purified IgM Fc5μ fragments to inhibit the binding of antibodies to leukocytes [44]. In carp, a suppression subtraction hybridization screen identified a γFc receptor from the kidney cells [45] and more recently a FcμR receptor was identified in catfish NK-like cells [46].

The large degree of phagocytic enhancement caused by opsonizing products belies the effectiveness of such a mechanism. However, phagocytosis may also occur in the absence of opsonization through the direct interaction of receptors on the phagocyte's surface with microbial or other ligands.

2.4.4 Enhancement of phagocytosis by other molecules

In salmon the ingestion of β-glucans by macrophages was mediated through both complement and a glucan receptor on the macrophage surface [47]. Although greater phagocytic activity was observed for opsonized β-glucan, native β-glucan was also ingested at a significant rate. This activity could be blocked by the pre-incubation of macrophages with a soluble β-glucan but only if the target was not already opsonized by complement [47]. Other studies using a variety of ligands have suggested the presence of mannose or scavenger receptors on fish phagocytes [36, 48]. In rainbow trout ingestion of protein coated beads was found to be independent of both IgM and complement, while pre-incubation with a scavenger receptor ligand inhibited phagocytosis. Treatment with cytochalasin (microtubule inhibitor), chloroquine (acts to increase lysosomal pH) and leupeptin (inhibits Thiol proteases) all prevented degradation of the bead's protein coat indicating that ingestion and breakdown occurred through the formation of phagolysosomes [36]. Although opsonization by Ig and/or complement may provide a more efficient method of targeting microbes for phagocytosis, in some systems depending on the nature of the pathogens, opsonin independent receptors may also play an important role. For example the phagocytic response of turbot phagocytes to *Microsporidium sp.* was not altered by the presence of Ig or complement. However, modification of sugars on the surface of the parasite reduced phagocytosis, suggesting a direct interaction between these sugars and a receptor expressed on the macrophages [49].

2.5 Respiratory burst

In mammals it has long been demonstrated that phagocytosis correlated to an increase in oxygen consumption by certain immune cells. The phenomenon has come to be known as the respiratory burst and was subsequently shown to correspond to the production of reactive oxygen species (ROS) [52]. Until recently this immune response was the single example of deliberately produced ROS and was shown to be regulated by the multi-component enzyme phagocyte oxidase (Phox), also known as NADPH oxidase [53]. The enzyme contains five essential protein subunits. Two subunits, gp91phox and p22phox, combine to form the membrane associated cytochrome b558 protein while the cytosolic components include p47 phox and p67phox. An additional component p40phox was shown to be associated with the oxidase but its function is unclear [54]. NADPH oxidase functions by transferring electrons from NADPH to molecular oxygen to produce superoxide, which results in the subsequent generation of secondary ROS [53]. It has been postulated that it is the reactive oxygen species themselves that directly affect the phagocytized microbe. However, Reeves et al. [55] suggested that the main function of ROS is to alter the pH environment within the phagosome causing the activation of proteases, which are toxic to the pathogens. The importance of NADPH oxidase for properly functioning immune response has been highlighted in individuals with chronic granulomatous disease. In this disease, the neutrophils of affected individuals have an impaired ability to kill microorganisms due to mutations or deletions that alter the Phox proteins [55].

NADPH oxidase is present in the plasma membrane of neutrophils, eosinophils and mononuclear phagocytes [54]. The most potent ROS response is mounted by the neutrophils. Developmental status of immune cells can affect the ROS response. For example, mononuclear phagocytes undergo a reduction in superoxide production following migration into tissues, where they undergo a final differentiation and become fully functional macrophages [56].

Before the respiratory burst response can be induced (triggered by PMA, for example) there is a requirement that the phagocyte must be primed by cytokines such as interferon-γ (IFNγ) [57]. Tsunawaki and Nathan [58], presented evidence that the mechanism behind the priming effect may be an increased affinity of the oxidase for NADPH. However, the priming response may involve other proteins, since the up-regulation of gene expression in particular the heavy chain of cytochrome b588, correlates with maximal priming of macrophages [59].

2.5.1 Fish respiratory burst response

The respiratory burst response has also been identified in fish phagocytes and was very similar to that observed in mammals. Direct evidence for the presence of NADPH oxidase in fish was provided by Secombes et al. [58] who used a spectroscopy method to identify a membrane component that shared characteristics with cytochrome b558 in rainbow trout macrophages. Furthermore, a polyclonal antibody against the carboxy terminal of human cytochrome b558 heavy chain was found to bind a membrane protein of eel and tilapia neutrophils [59-60]. The synthetic peptide that corresponded to the C-terminus of the heavy chain of cytochrome b558 was able to block ROS in neutrophils in a dose dependant manner [60]. Shiibashi and Iida [61], demonstrated that similar to mammalian NADPH oxidase the superoxide generating enzyme of tilapia neutrophils required NADPH and NADH to serve as electron donors. The most definitive evidence for the presence of NADPH oxidase in fish has been the molecular cloning and sequencing of all five subunits of the enzyme from the Japanese pufferfish [62]. The two subunits, gp91phox and gp22phox, *that form the membrane bound cytochrome b558* share 68.0 and 61.8% identity with the human homologues, respectively.

Complementary to the relatively recent progress that has been made in the biochemical characterization of the respiratory burst response in fish was a more long standing endeavor to advance the understanding of the regulatory mechanisms of the ROS response in fish. Due to the paucity of available recombinant fish cytokines, crude preparations obtained through the collection of supernatants from mitogen stimulated leukocyte cultures were used. Employing a goldfish model system, investigators in our lab showed that these crude macrophage activation factors (MAF) preparations contained soluble components that have the ability to activate or alternatively deactivate fish macrophages, as shown by the production of reactive oxygen and nitrogen intermediates by activated macrophages [63-65]

In trout, pervious investigation had identified the macrophage activating factors (MAF) contained within mitogen stimulated cell supernatants to be a product of T-cells [66]. Crude cytokine preparations of this kind were used to induce the ROS of rainbow trout macrophages *in vitro* [67]. The priming ability of MAF in this study was found to synergize with lipopolysaccharide (LPS) and human recombinant TNFα to enhance the ROS. Mulero and Meseguer [68], used MAF to enhance the ROS of gilthead seabream leucocytes. They identified a synergistic effect when MAF was co-incubated with LPS for 48 hr that enhanced the nitric oxide response. However, this combination of MAF and LPS had the effect of inhibiting the respiratory burst response, which was in contrast to what has been reported for trout macrophages. The observed inhibitory effect of ROS response of seabream macrophages by MAF and LPS may be explained by the earlier findings reported by our laboratory on the ROS kintetics of a goldfish macrophage cell line (GMCL). In the GMCL, the maximal priming of the respiratory burst occurred after only 6hrs following treatment with MAF, and was reduced 24 to 48 hr later. This differed from the kinetics of LPS-induced ROS which steadily increased over the 48 hr. A synergistic effect of MAF and LPS was observed but this increased priming followed the kinetics of MAF alone which meant there was little priming observed after 48 hr [64].

If we are to develop a fully functional concept of inflammation in fish, our knowledge of the kinetics of the ROS of fish phagocytes would be critical for the modulation of this response for the protection against the invading pathogens. An important aspect of the ROS response in fish was the reported differential ability of different developmental stages of phagocytes to respond to pathogens. Comparisons of the activation response of monocytes and mature macrophages from goldfish have revealed some distinct differences in the priming of the ROS. Monocytes treated with MAF and LPS demonstrated a rapid but transient response within 6hrs that was greatly reduced after 48 hr. The response of mature macrophages was less rapid and instead there was a gradual increase in priming over the course of a 48 hr cultivation period [69]. The priming kinetics of mature goldfish macrophages were consistent with observations from studies of both mammals and other fish species where macrophages generally require a minimum incubation period of about 48 hr to be primed [67-70].

Recently, the investigations of the mechanisms of activation of phagocytes have shifted from the use of relatively undefined substances such as MAF to the use of recombinant cytokines. One cytokine of particular interest with regards to the ROS is IFNγ [55]. IFNγ homologues have been recently identified in catfish, fugu and zebrafish but functional analyses are yet to be performed [71-73].

2.6 Nitric Oxide

Complementing the ROS activity of phagocytic immune cells is an alternate system of reactive antimicrobial molecules comprising the nitric oxide (NO) response. The production of NO, a broadly featured signal/effector molecule, is mediated through a family of nitric oxide synthase enzymes (iNOS, eNOS, nNOS). These include constitutively expressed forms (nNOS and eNOS) and an inducible form (iNOS) which is responsible for catalyzing the NO response of activated phagocytes. iNOS catalyzes the breakdown of L-arginine into citruline which results in the production of NO. The induction of iNOS in mammals is regulated by cytokines such as TNFα, IFNγ , IL-1, IL-4 and TGFβ [74].

2.6.1 Fish nitric oxide response

The first inducible nitric oxide synthase was identified in the goldfish [75] and further studies by Liang and colleagues [76] have identified iNOS in rainbow trout from the head kidney and gill tissue of animals infected with the bacteria *Renibacterium salmoninarum*. Trout iNOS amino acid sequence shared an 85% homology with a partial sequence obtained from goldfish and a relatively high (69-71%) identity with mammalian iNOS. Tissue expression of trout iNOS was found to be significantly higher in bacterially challenged fish as compared to the controls. The stimulation with LPS was found to up-regulate the expression of iNOS in trout head kidney macrophages [76].

Our laboratory was first to demonstrate the ability of LPS and MAF to induce the NO response in fish macrophages [77-78]. This response was inhibited by the addition of N^G-MMLA suggesting that the metabolic pathways for the production of NO in fish were similar to those of mammals [63]. Co-stimulation of cells with both LPS and MAF also appeared to have a synergistic effect that further enhanced the NO response [63]. Similar to the ROS response, the peak production of NO was dependent on the maturation state of goldfish macrophages [63]. For example, goldfish monocytes were unable to mount a significant NO responses while mature macrophages were potent producers of NO [69]. Human monocytes have been noted to acquire the ability to produce NO only after several days in culture [79-80].

Identification of the specific factors involved in the NO response has been facilitated by the availability of purified/recombinant immune mediators. In turbot, LPS in combination with a turbot IFNαβ-like molecule induced the NO response in cells otherwise non-response to LPS alone. However, this stimulatory capacity was not present in all macrophage subpopulations. Human recombinant TNFα when combined with LPS was able to induce a significant enhancement of the NO production of all macrophage subpopulations in turbot [81]. Cells treated with pentoxifylline an inhibitor of TNFα (but not iNOS) were found to have significantly reduced NO production compared to controls [82]. More recently, our laboratory identified a novel inducer of the NO response in macrophages, the iron-binding host protein called transferrin. Cleaved products of transferrin were shown to induce potent NO response in goldfish macrophages. Interestingly, recombinant goldfish transferrin N- and C-lobes also induced the NO production in a mouse macrophage cell line indicating that this mechanism of activation may be highly conserved [83].

Gilthead seabream vaccinated against the pathogenic bacterium *Photobacterium damselae* have significantly higher levels of NO production than non-vaccinated individuals *in vivo* and *in vitro* [84]. This heightened response correlated with greater protection from subsequent bacterial challenge. Using the seabream/*P. damsela* model Acosta et al. [84] reported that blocking the NO response using the iNOS inhibitor L-NAME significantly increased the susceptibility of fish to infection. Similarly, the bactericidal activity of catfish phagocytes against *Aeromonas hydrophila* was enhanced following vaccination and this bactericidal activity was partially blocked by the addition of N^G-MMLA another inhibitor of the NO pathway [70]. These authors also reported that the supernatants from immunized cell cultures exposed to the vaccinating strain induced greater NO response than supernatants collected from cells that were stimulated with a different bacterium [70]. Findings such as these demonstrate the importance of the NO response in the resistance of fish to certain pathogens.

2.7 Pro-Inflammatory Cytokines of Fish

The understanding of the cytokine regulation of the immune response in the fishes has seen a great deal of recent advancement due to the cloning of a number of cytokines in fish. Of particular interest with regards to leukocyte biology have been the pro-inflammatory cytokines, interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNFα), interferon gamma (INFγ), and transforming growth factor beta (TGFβ). The fish homologs of these molecules affect a wide spectrum of inflammatory activities including chemotaxis, phagocytosis as well as respiratory burst and the nitric oxide response.

2.7.1 Tumor necrosis factors

The tumor necrosis factor family of ligands includes several potent pro-inflammatory cytokines. In mammals the members of this family include, TNFα, TNFβ (LTα) and lymphotoxin-β (LTβ). Perhaps the best characterized member, TNFα, is a type II trans-membrane protein that can be active in its membrane bound form but also can be cleaved to release a soluble peptide [4]. In mammals, TNFα and TNFβ are located in tandem with the MHC locus [4].

The identification of TNFα in mammals came with the observation that cells responding to bacterial signals produced by a factor capable of causing necrotic death of some tumors. Although it did not turn out to be a suitable treatment for most cancers, TNFα has been shown to play a role in necrotic and apoptotic cell death, cellular proliferation, development as well as inflammation [85]. TNFα has also been implicated in a number of diseases and medical conditions such as rheumatoid arthritis, asthma and septic shock [85].

In mammals the production of TNFα by macrophages has been reported to be induced by LPS as well as cytokines such as IFNγ [86]. TNFα may be produced in multiple isoforms due to post translational or transcriptional modification. Different isoforms have been shown to be produced depending on the initial stimulus such as LPS or other factors such as granulocyte macrophage colony stimulating factor (GM-CSF) [87].

During inflammation, signals detected by pathogen recognition receptors (PRR) initiate the expression of TNFα by macrophages and endothelial cells. This sets in motion a cascade of responses involving various cytokines, chemokines, adhesion molecules and recruited immune cells. The reproduction of these events by the injection of TNFα, and the reduced inflammatory response observed in TNF deficient individuals highlights the central role played by this cytokine [88].

The primary receptors for TNFα are the TNF receptors 1 and 2 (TNFR1 and TNFR2). The majority of the physiological effects of TNFα occur through TNFR1 which possess a intracellular death domain that was shown to be involved in signaling [89]. Absence of TNFR1 leads to a severe increase in susceptibility to bacterial and protozoan infections. Absence of TNFR2 can result in elevated LPS induced serum TNFα levels suggesting a regulatory role for this receptor [89].

Initial work that examined the functional properties of stimulated fish leukocyte supernatants found the ROS response and migration of trout leukocytes could also be induced by mouse TNFα, suggesting species cross-reactivity of the molecule [90]. This was confirmed by the observation that anti-TNF antibody but not an anti-IL-1 antibody blocked the induction of ROS [90].

Subsequently, fish TNF sequences that share greatest similarity to mammalian TNFα have been identified in a number of species including Japanese flounder [91], trout [92], carp [82, 93], catfish [94], seabream [95-96] and turbot [97]. In species such as flounder and catfish, TNFα was encoded by a single gene [91, 94], while in other fish, notably trout and carp, several isoforms have been described [82, 93]. Functionally, both trout isoforms appear to have an equivalent effect for induction of chemotaxis and phagocytosis. However, distinct functional properties have been described for different isoforms. For example, TNFα-1 induced further expression of TNFα-1 but not TNFα-2, while TNFα-2 stimulated the expression of both TNFα-1 and 2, suggesting its role in cross-isoform regulation [98]. It is possible that a third isoform identified in carp may be functionally distinct from the other two by having a distinct pattern of tissue expression [93].

Mitogens or microbial molecules such as LPS induce significant production of pro-inflammatory cytokines, including TNFα, by both fish and mammalian leukocytes. In catfish, carp, flounder and trout stimulation of leukocytes with bacterial endotoxin (LPS) dramatically up-regulates TNFα expression [82, 91-92, 94, 99]. In trout it was observed that although both monocytes and mature macrophages were capable of up regulating TNFα mRNA in response to LPS, the induction was significantly higher for mature macrophages (40 fold above control) compared to monocytes (6 fold above control) [99]. This suggests a role for mature resident tissue macrophages in the early TNFα response and a central role for TNFα during the initiation stages of the inflammatory response in fish [98].

In addition to LPS, TNFα mRNA expression was also up-regulated in macrophages treated with 12-phorbol 13-myrstic acid (PMA) and concanavalin A (Con A) in the flounder and catfish [91, 94]. *In vivo* challenge with live bacteria, viruses, protozoa and helminth parasites, induces an increase in the mRNA expression of TNFα [82, 97, 100].

There is evidence suggesting that the transcriptional regulation of fish TNFα (particularly in response to LPS stimulation) was similar to that of mammals in that nuclear factor(NF)-κB pathway appears to be involved [101], since the addition of a NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) down-regulated TNFα expression in LPS stimulated carp leukocytes [82]. Further analysis of the Japanese flounder TNFα promoter region identified NF-κB and interferon regulatory factor (IRF) transcription factor binding regions [101].

TNFα has been implicated in the regulation of almost every aspect of the fish inflammatory response including up regulation of other inflammatory mediators, chemotaxis, phagocytosis, and production of reactive oxygen and nitrogen intermediates by phagocytes. Recombinant trout TNFα has been shown to induce chemotaxis and phagocytosis *in vitro* as well as up regulation of the cytokine IL-8 which is known to have chemoattraction properties [98]. In addition, intraperitoneal injection of recombinant TNFα has been shown to induce the migration of neutrophils into the peritoneal cavity of turbot [97].

In seabream, intraperitoneal injection of recombinant TNFα primed the ROS in peritoneal exudate cells and head kidney leukocytes [95]. Turbot recombinant TNFα failed to trigger a ROS but did have a significant effect on NO production after 72 hr of incubation [97]. The addition of pentoxifylline (PTX), a TNFα inhibitor, abrogated the NO response of leukocytes to LPS [82]. PTX also inhibited proliferation of carp leukocytes [82], and the injection of TNFα induced proliferation of seabream head kidney leukocytes [95], suggesting that TNFα may promote the proliferation of fish immune cells.

The vast majority of TNF-like sequences identified in fish appear to be more similar to TNFα. Subsequent efforts to identify a TNFβ (LTα) gene in fish failed, leading some researchers to suggest that the TNF gene of fish was ancestral and that the gene duplication event that gave rise to the mammalian α and β forms occurred later in evolution [102]. However, Saven et al. [102], suggested that a novel TNF sequence they had identified in fugu and zebrafish (TNF-N) may be similar to the elusive teleost TNFβ. This conclusion was based on the arrangement of a TNF locus in fish that had TNFα and TNF-N existing in tandem and in the same transcriptional orientation [102]. Ultimately, the authors had to stop short of calling TNF-N "TNFβ" because of very low sequence homology.

A subsequent study in trout reported on the presence of a LTβ (not the same as TNFβ which is referred to as LTα) in trout [103]. LTβ is not expressed by mammalian macrophages but rather B and T cells [103]. Phylogenetic comparison of the trout LTβ genes with the TNFN sequences and the mammalian LTβ indicate that fish TNFN may be a LTβ-like molecule and not TNFβ [103].

The effect of both TNFα and LTβ ligands are mediated through the TNFR-1 and 2 receptors. Both receptors have been cloned in Japanese flounder [104]. TNFR-1 and 2 are expressed on the surface of flounder leukocytes and can be up-regulated by stimulation from LPS, Con A, or PMA [104]. TNFR-1 appeared to be more highly expressed in leukocytes compared to TNFR-2 and shared the same timing of response to LPS as Japanese flounder TNFα. Mammalian TNFα was shown to be primarily recognized by TNFR-1 [104].

2.7.2 *Interleukin 1β*

Interleukin-1β is a highly pleiotropic pro-inflammatory cytokine produced by macrophages. IL-1β in mammals has been shown to regulate fever, cytokine release by T-lymohocytes (e.g. IL-2) as well as the NO response [105]. Similar to TNFα, IL-1β like activity was identified in activated fish leukocyte supernatants before the first teleost IL-1β sequences were identified. The first indication that an IL-1-like cytokine exists in fish was the observation that PMA pre-stimulated carp macrophages and neutrophils exhibited a significant proliferation effect on an IL-1-dependent murine T-cell line. This effect was inhibited by the addition of an anti-IL-1 antibody [106]. Since then IL-1β like sequences have been identified in carp [107], trout [108], seabass [109] and catfish [110].

Up regulation of teleost IL-1β was observed in response to recombinant IL-1β and TNFα [100, 108] as well as LPS [108-109, 111], PMA [111] and phytohemaglutinin (PHA) [112]. The addition of a NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) to LPS-treated head kidney leukocytes abrogated IL-1β mRNA expression suggesting that like TNFα, NF-κB was important for IL1β biological activity [111]. *In vivo*, intraperitoneal injection of recombinant IL-1β caused increased expression of IL-1β and lysozyme II in the head kidney and gills of trout [113], while catfish infected with bacteria exhibited elevated expression levels of IL-1β [110].

The cleavage of mammalian IL-1β by caspase to release the mature biologically active molecule was dependent on the presence of an ICE cut site. However, fish IL-1βs all appear to lack a clear ICE cut site like that of mammals [108]. To determine whether fish IL-1β was still cleaved before release from the cell, a trout macrophage cell line was transfected with a vector carrying a HIS tagged pro-IL-1β sequences [114]. Western blot analysis identified both the cleaved and non-cleaved forms of the molecule in the cell lysates, but only the cleaved form was found in the supernatants [114]. This suggested that trout IL-1β like its mammalian counterparts must be cleaved by some mechanism before its release from cells.

IL-1β produced through the recombinant expression of the C-terminal portion of the molecule has been found to be biologically active. Recombinant trout IL-1β induced chemotaxis of leukocytes *in vitro* [120] and *in vivo* [113, 115]. The phagocytic response was similarly up-regulated [108, 113, 116]. The proliferation of fish leukocytes was also enhanced by rIL-1β in both carp and trout [108, 112]. A bioactive IL-1β derived peptide was shown to increase the bactericidal activity of trout macrophages, but the killing was not mediated through super oxide production [116]. Lastly, injection of recombinant IL-1β was shown to protect carp against bacterial challenge with *Aeromonas hydrophila* [109] and trout challenged with *Aeromonas salmonidicida* [113].

Both IL-1 receptor 1 (IL1-R1) and IL-1 receptor 2 (IL1-R2) have been cloned in fish [117-118]. IL1-R1, which was shown to be primarily responsible for mediating the activity of IL-1β, was found to be up-regulated in salmon gill, liver, spleen and head kidney following LPS stimulation [118]. IL1-R2 was shown to be predominantly expressed on B-cells, neutrophils, and monocytes and functions as a regulator of IL1-β activity. Trout IL1-R2 was shown to be up-regulated in head kidney leukocytes following stimulation with LPS and TNFα [117].

2.7.3 Interferons

The interferons (IFN) are an important group of cytokines that are involved in regulating host defense against pathogens [119]. This family of cytokines includes both type I (IFNα/β) and type II (IFNγ) molecules. The type 1 interferons are induced by viral signals such as double stranded RNA (dsRNA) and their production by cells signals to the surrounding cells to begin production of antiviral proteins such as Mx protein [119]. The type 1 interferons have been identified in zebrafish [120], Atlantic salmon [121] and catfish [122], and all demonstrate sensitivity to viral stimulants such as Poly I:C or UV killed dsRNA viruses. Production of these IFN molecules greatly increased the antiviral activity of the cell supernatants [120-122] and both zebrafish and salmon type 1 interferons were shown to induce the production of the antiviral Mx protein [120-121].

The type 2 interferon, IFNγ, plays an important pro-inflammatory role since it regulates the antimicrobial responses of immune cells. In mammals, natural killer (NK) cells secrete IFNγ following co-stimulation of the cells with cytokines IL-12 and IL-18 [119]. IFNγ has been shown to activate macrophage and up-regulate the killing machinery of the cells through the induction of NADPH oxidase and/or iNOS [119]. IFNγ has been shown to regulate CXC chemokine production as well as antimicrobial compounds such as the guanylate binding protein [119].

In teleosts, IFNγ has been recently identified in Fugu [72], zebrafish [73] catfish [71] and trout [123]. There is very little information about functional properties of teleost IFNγ. Zebrafish IFNγ has been shown to be up-regulated in response to Poly I:C and catfish IFNγ was produced by NK cells, like in mammals [71, 73]. More recently a recombinant trout IFNγ was shown to induce the expression of a guanylate-binding protein in macrophages [123]. The availability of a recombinant IFNγ will allow for the characterization of the functional role of this cytokine in teleosts. Many of the pieces making up the overall picture of IFNγ activity in fish are already in place and only await the connections to be made through functional studies. The cloning of NADPH oxidase components [62] and the presence of NK cells in fish [124] as well as the identification of IFNγ inducible proteins such as guanylate binding protein [123], will allow for direct comparison between mammalian and fish IFNγ functions. Furthermore, the cytokines IL-12 and IL-18 responsible for the induction of IFNγ production by NK cells have also been identified in fish [125-126].

IL-12 which is formed by the combination of two subunits encoded on different genes was recently identified in fugu [126]. Expression of the p35 subunit of IL-12 increased in the head kidney and spleen of fugu following Poly I:C injection. The p40 subunit was constitutively expressed and was not up-regulated by Poly I:C, suggesting p35 plays a greater role in regulation of IL-12 production [126]. IL-18 was identified in trout and was expressed in two alternatively spliced forms (IL18-A and B) [125]. Both forms were expressed in both a macrophage cell line and head kidney leukocytes. Stimulation with LPS, Poly I:C, or IL-1β all failed to induce the mRNA expression [125].

The type 1 and 2 interferons signal through different receptors referred to as the type 1 interferon and type 2 interferon receptors,respectively, although there are some similarities in their intracellular signaling as both utilize the Jak-stat pathway [127]. Interferon receptors have not been characterized in fish. The type 1 receptor was not present in the predicted gene cluster of pufferfish suggesting it may not exist in fish [128]. The IFNγ genes between species share very low homologies suggesting a co-evolution with the receptors in a manner that may be in part tailored by specific pathogens [119].

2.7.4 *Transforming growth factor beta*

The transforming growth factor β (TGFβ) family is a group of pleiotropic multifunctional proteins. TGFβ plays an important role in tissue remodeling and hematopoeisis as well as inflammation since it is known that this cytokine has immunosuppressive and anti-inflammatory properties in mammals [129].

In contrast to the mechanisms of activation of fish phagocytes, much less work has been done to characterize deactivation of phagocytes, an important process required for down-regulation of inflammatory responses. Our lab was first to demonstrate the presence of a macrophage deactivating factor in the supernatants of culture goldfish macrophages measured by the inhibition of the NO response [130]. A 15kD protein present in macrophage culture supernatants was responsible for this deactivation response, and based on the size of the molecule, the authors speculated that it may be a fish equivelent of TGFβ [130]. In rainbow trout, a recombinant bovine TGFβ was shown to reduce the ROS of activated macrophages [131]. Bovine TGFβ was also able to inhibit pro-inflammatory signals present in MAF preparations [131]. These results suggest that TGFβ binding region may be highly conserved.

To date TGFβ sequences have been reported from several fish including trout [132], carp [133], and seabream [134]. Unfortunately, the functional activity of any of these transcripts has yet to be examined. The identification and characterization of the

TGFβ receptor system in fish remains to be determined. However, the primary mechanism of signaling which occurs through the formation of a heterotetracomplex of type 1 and 2 serine-threonine kinase receptors appears to be conserved through the metazoans [129].

2.8 Conclusion

The study of the mechanisms of inflammation in fish is in infancy when compared to the significant body of information available for different mammalian systems. The basic framework for a functional inflammatory response in fish appears to be very similar to that of mammals; fish possess functional phagocytes that are similar to those of mammals and have a panel of pro-inflammatory cytokines and other molecules that mediate a plethora of inflammatory events. As we develop phagocyte cell lines and fully characterize the pro-inflammatory cytokines of fish, we will be able to address the precise mechanisms involved in antimicrobial responses of fish phagocytes, the regulation of inflammatory response and tissue repair events in fishes.

CHAPTER 3

MATERIALS AND METHODS

3.1 Fish

Goldfish were obtained from Mt. Parnell Fisheries Inc. (Mercersburg, PA) and maintained at the Aquatic Facility of the Department of Biological Science, University of Alberta. The fish were kept at 20°C in a flow through water system and fed to satiation daily with trout pellets. The fish were acclimated to this environment for at least three weeks prior to use in experiments.

3.2 Culture medium

The culture medium for goldfish macrophages has been described previously [63]. Complete medium contained 5% carp serum and 10% newborn calf serum (NCS; Hyclone, Logan, UT).

3.3 Isolation and cultivation of primary kidney macrophages

Isolation of goldfish kidney leukocytes and the generation of primary kidney macrophages (PKM) were performed as previously described [63]. Briefly, goldfish macrophage cultures were established by seeding freshly isolated kidney leukocytes (18-20×10^6 cells flask^{-1} from individual fish) into 75 cm^2 tissue culture flasks containing 15 mL of complete medium and 5 mL of cell-conditioned medium (CCM) from previous cultures. PKM cultures were incubated at 20°C until the cells were at a stage of active proliferation (5-6 day old cultures). Whole macrophages cultures containing all three subpopulations, or macrophage subpopulations sorted into early progenitor, monocyte and mature macrophage populations using a FACS Calibur flow cytometer (Becton/Dickinson), were used to conduct expression analyses and functional studies.

3.4 Identification of goldfish prominin sequence

3.4.1 cDNA library screening and isolation of goldfish prominin transcripts

Clones encoding for goldfish prominin were identified from a differential cross screen that looked to identify differentially expressed genes in proliferative and senescence phase goldfish primary macrophage cultures [135]. The PCR amplified clone inserts corresponding to each of the confirmed prominin positive clones were purified using the QIAquick PCR purification kit (Qiagen), and sequenced using a DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia) and a PE Applied Biosystems 377 automated sequencer. Single-pass sequences were analyzed using Genetool (Biotools Inc.) software and subsequent gene annotations were conducted using NCBI GenBank BLAST programs.

3.4.2 5' RACE

A 1462 bp sequence was obtained from the library screen which encoded for the 3' end of the protein including the stop codon. A SMART RACE cDNA Amplification kit (BD Biosciences, Palo Alto, CA) was used according to the manufacturer's protocol to obtain the complete ORF. Briefly, 5′-RACE-Ready cDNA was generated from 500 ng of mRNA extracted from un-stimulated goldfish macrophages. 5′-RACE was performed as per manufacturer's protocol using an antisense gene-specific primer (GCTGCAGGCCCACAACGACCC) based on the insert sequence.

The fragments generated by 5′-RACE were subcloned into the pCR, 2.1-TOPO vector and sequenced. A single 5′-RACE subclone was sequenced by designing primers to the new portions of the gene as more of the gene sequence was generated. Sequence fragments were assembled using Genetool (Biotools, Inc.). After obtaining the complete ORF through RACE, the full length transcript was PCR amplified and sequenced 3 times. All DNA sequencing was performed using the DYEnamic ET-Terminator Cycle Sequencing Kit (Amersham-Pharmacia Biotech) and Applied Biosystems 377 DNA sequencers according to standard manufacturer-supplied protocols.

3.4.3 In silico analyses

Sequence manipulations were performed using Genetool (Biotools Inc.) software package. BLAST searches of the NCBI GenBank database were conducted to identify related sequences. Hydropathy analysis was conducted using TMpred software (http://www.ch.embnet.org/software/TMPRED_form.html) and the signal peptide cleavage site was predicted using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). N-glycosylation sites were predicted by the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

3.5 Quantitative PCR analysis of goldfish prominin expression in tissues and sorted goldfish macrophages

The tissue expression of goldfish prominin was analyzed using a real time RT-PCR system (7500 real time PCR system, Applied Biosystems). RNA was isolated from the kidney, gill, liver, spleen, heart, brain and intestine of 5 fish and the first-strand synthesis was done using 4 μg total RNA, random hexamers (Invitrogen) and SuperScript™II RT (Invitrogen) according to manufacturer's protocols.

Expression levels were compared using the relative Ct method and as such an initial validation experiment was conducted to ensure that prominin and the endogenous control β-actin had equivalent PCR efficiencies. Following the validation experiment the relative quantification experiment was conducted under these conditions: 95°C for 3 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. Melting point analysis was conducted for each reaction. The relative difference between the target (prominin) versus the endogenous control (β-actin) threshold Ct values were then calculated and arithmetically converted into fold differences as compared to the chosen calibrator kidney. The primers used are reported in Table 3.1.

Quantitative RT–PCR was also performed on sorted *in vitro*-derived goldfish kidney macrophage subpopulations (early progenitors, monocytes and mature macrophages) using a FACS Calibur flow cytometer (Becton/Dickinson). The PCR conditions were the same as described in the previous paragraph. In addition, standard PCR was conducted for visualization on a gel. First-strand synthesis was done using an oligo(dT) primer (Invitrogen), 1μg total RNA and SuperScript™II RT (Invitrogen) according to manufacturer's protocols.

PCR Amplification was performed using the following cycling parameters: 94 °C for 3 min; 27/28 cycles of 94 °C for 35 s; 56/62 °C for 40 s; 72 °C for 2 min 30s. Once removed samples were immediately placed on ice. For the purposes of this PCR it was determined that 28 cycles was within the linear range for goldfish prominin. The annealing temperature for the prominin primers was 62°C. For the β-actin loading control 27 cycles and an annealing temperature of 56°C were appropriate. The goldfish prominin and β-actin specific primers are reported in Table 3.2.

3.6 Southern blot analysis of goldfish prominin

Genomic DNA was isolated from goldfish erythrocytes using the DNeasy Tissue Kit (50) from Qiagen. DNA was incubated with the restriction enzymes Kpn I, Sph I, and Xba I overnight. Kpn I and Xba I both had no predicted cut sites within the probe. Sph I had one cut site situated very near to one end of the probe, The DNA was then run on a 1% agrose gel and transferred using a high salt transfer buffer onto a nitrocellulose membrane (BioRad). Briefly, the gel was depurinated (0.25M HCl), denatured (1.5M NaCl, 0.5M NaOH) and neutralized (1.5M NaCl, 5M TrisCl pH 7.0) before the DNA was transferred onto the membrane using a standard blotting apparatus and 20X SSC. The blot was UV cross linked and blocked before being probed with a P-32 labeled product of ~1200 bp. Probed blots were washed under conditions of moderate stringency and film was exposed for 24 hours at -80°C.

3.7 Identification of goldfish tumor necrosis factor alpha sequence

3.7.1 Obtaining the goldfish TNFα sequence

Degenerate primers designed against tumor necrosis factor alpha (TNFα) in carp were used to obtain partial sequences which were blasted against the NCBI database and identified as the likely homologs of goldfish TNFα-1 and 2. Using the same protocol as out lined in section 2.4.2 RACE PCR was conducted based on the partial sequences and the subsequent RACE products were sequenced. The full ORF for goldfish TNFα-2 was obtained however for TNFα-1 only the portion representing the cleaved active fragment was obtained.

3.7.2 In silico analyses

Goldfish TNFα types one and two were first identified as such by blasting the sequences against the NCBI GenBank database. The goldfish sequences shared very high homology with those already described from carp and were therefore denoted as type 1 or 2 based on the carp nomenclature. Nucleotide alignments of goldfish TNFα-1 and 2 to each other as well as amino acid alignments with TNFα sequences from other fish species and mice were conducted using the Clustal W software (http://www.ebi.ac.uk/clustalw/). Phylogenetic analysis was conducted with Clustal X software using the neighbor joining method and bootstrapped 1000 times.

3.8 Expression of recombinant goldfish TNFα-2 using prokaryotic systems

3.8.1 Generation of PCR product and cloning into expression vector

The portion of the TNFα-2 sequence representing the cleaved active fragment was PCR amplified from kidney cDNA using primers designed to meet the requirements of the pET SUMO expression vector (Invitrogen). The expression primers were: sense 5'-ATGTCCAAGGCCGCGATCCATTTA-3'; antisense 5'-CTAAAACACCCCGAAGAA-3'. The resulting 462 bp PCR product was cut out and gel purified using the QIAquick gel extraction kit (Qiagen) before cloning. As per the manufacture's protocol, the gel purified product was ligated into the pET SUMO vector and the resulting construct was transformed into competent *E. coli* (One Shot Mach1-T1). After transformation the cells were plated onto LB-kanamycin plates and incubated overnight at 37°C. A random selection of colonies were picked and a colony PCR was conducted using vector specific primers in order to identify positive clones. Isolates identified as positive were then grown up in a milliliter of LB-Kanamycin and a mini-prep was performed using the QIAquick Spin miniprep kit (Qiagen). To determine that insert was in the appropriate orientation and in frame the purified plasmids were sequenced using the vector specific primers.

3.8.2 Transformation and pilot expression of recombinant goldfish TNFα in E. coli

A plasmid containing the in frame TNFα insert was transformed into BL21 Star One Shot *E. coli* (Invitrogen) and grown at 37°C over night in 10mL of LB-Kanamycin. Pilot expression was conducted by inoculating 10 mL of LB-Kanamycin with 250 μL of the overnight culture and incubating at 37°C for 2 hr. After the culture had reached the mid-log growth phase (O.D.$_{600}$ ~0.5-0.8) isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a concentration of 0.1 mM. At this point and at 2, 4, and 6 hrs post induction a 500 μL sample was taken from the culture and spun down at 14 000 rpm in a micro-centrifuge for 20 s. The pellets and supernatants were separated and stored at -20°C and 4°C respectively. The samples were then analyzed by SDS-page and Western blot using an anti-His antibody to detect expression of the recombinant protein (section 2.8).

3.8.3 Large scale expression and purification of recombinant TNFα

Maximal induction of the recombinant protein was found after 4 hrs. To scale up production 10 mL of overnight culture was added to 1 L of LB-Kanamyin media and grown for 2 hr as 37°C before the addition of IPTG to a final concentration of 0.1 mM. The culture was then returned to 37°C and grown for 6 hr. Our pilot expression indicated that the majority of the protein was found to be in the insoluble fraction and thus required isolation from the bacterial occlusion bodies. Therefore, the resulting culture was then spun down in a Sorvall centrifuge at 4810 x *g* for 15 min in order to pellet the bacteria. After centrifugation the supernatants were removed and the pellets were placed at -20°C overnight. The following day 50 mL of lysis buffer (5 mL of 10x FastBreak cell lysis reagent (Promega) into 45 mL of denaturing wash buffer 100 mM Hepes, 10 mM Imidazole, 7 M Urea, pH 7.5) was added per 500 mL culture pellet and allowed to mix with gentle rocking for 20 min. The subsequently lysed bacteria were then incubated with 1 mL of MagneHis Ni-Particles (Promega) for 15 min before being separated using the PolyATtrack System 1000 magnet (Promega). The supernatants were discarded and the beads were washed 3 times for 2 min under denaturing conditions using the denaturing wash buffer described above. Following the washes the recombinant protein was eluted from the beads using the wash buffer with a high concentration of Imidazole (500 mM). Eight 1 mL elution fractions were collected and analyzed by SDS-page and Western blot using an anti-His antibody to detect the presence of the recombinant protein (section 2.8). The appropriate size band was observed in all 8 elution fractions. The first fraction was discarded as it appeared to contain some amount of non specific products. The remaining fractions were pooled and dialyzed overnight in 1x PBS using SnakeSkin Pleated Dialysis Tubing (Pierce) in order to reduce Imidazole and Urea to negligible concentrations. The protein was renatured in 10 volumes of 40 mM Tris (pH 8) overnight before being further dialyzed in 1x PBS for 6 hours. To concentrate, dialysis tubing was placed in polyethylene glycol flakes for 4-5 hrs. LPS removal was achieved using the EndoTrap Red endotoxin removal column (Cambrex) as per the manufacturers directions. Finally the protein concentration of the final sample was determined using a Micro BCA Protein Assay Kit (Pierce) and the presence of the recombinant in the sample was again verified by western blot.

3.9 Detection of recombinant protein by SDS-page and Western blot

Samples were run on a reducing SDS-page 12% polyacrylamide gel to separate proteins by size. The protein was then transferred onto nitrocellulose membrane (BioRad) and stained with Ponceau S solution (Sigma-Aldrich) to visualize the standard. The standard was cut off and the nitrocellulose membrane was then blocked with 0.5% BSA dissolved in TTBS (100 mM Tris-HCL, 9% NaCl, 0.1% Tween 20, pH 7.5) for 30 min before incubation at 20°C with the primary antibody overnight. Because the pET SUMO appends on an N-terminal 6xHis tag, the recombinant protein can be detected with a mouse anti-His antibody (1:5000). Following incubation the primary anti-His antibody was washed three times for 10 min in TTBS and a secondary goat anti-mouse antibody (1:1500) was added. After 1 hr the secondary was removed and the blot was again washed three times in TTBS followed by three 10 min washes in TBS (100 mM Tris-HCL, 9% NaCl, pH 7.5). Bound antibody was then detected using a chromogenic BCIP/NBT development kit (BioRad).

3.10 Chemotactic response of goldfish macrophages to recombinant TNFα

Cells were isolated from goldfish kidneys and cultured until day 6 (section 2.3). Cultures from 6 fish were spun down at 200 x *g* for 10 min and re-suspended in 500 μL of complete media. For each treatment, the agent tested was added to the lower well of a leucite chemotaxis chamber (Nucleoprobe Corp.) while 5 x 10^5 cells were placed in the upper well. The upper and lower wells were separated by polycarbonate membrane filters (5μm pore size; Nucleopore Corp.). The small size of the pores required active translocation of the cells preventing passive movement from the upper to lower wells. The total volume in the upper or lower well was 150 μL. The chambers containing the cells were incubated for 4 hr at 20°C to provide sufficient time for cells to respond to the chemical gradient. Following the incubation, the contents of the top wells were aspirated, the filters were than removed, the top-sides were washed then mounted bottom-side-up on microscope slides, air dried and fix-stained using Wright's solution. Chemotactic activity was determined as the total number of cells counted in twenty randomly selected fields of view at 100x magnification. In order to measure chemokinesis, equal concentrations of either TNFα or LPS were added to both the upper and lower wells. The treatments were as follows: complete media, LPS (5ng/well), rTNFα (0.001, 0.01, 0.1, 1, 10,100, and 1000 ng/well). For the chemokinesis experiments 10 ng of recombinant TNFα or 5 ng of LPS, where added to upper and lower wells, respectively. To determine an optimal concentration for the positive control LPS a chemotaxis assay was conducted as described for a range of LPS concentrations.

Statistical analysis was conducted by an analysis of variance (ANOVA). Difference in means between treatments was determined by Tukey's test.

3.11 Phagocytic response of goldfish macrophages to recombinant TNFα

Cells were isolated from goldfish kidneys and cultured until day 6 (section 2.3). Cultures from 6 fish were spun down at 200 x *g* for 10 min and re-suspended in 500 μL of complete media. Phagocytosis assays were carried out in 96 well plates with 3 x 10^5 cells per well at a final volume of 100 μL/well. Cells were treated with complete medium, MAF or rTNFα at various concentrations and incubated at 20°C with fluorescent beads (2.0 μ diameter YG, Polysciences) for 16 hr. Following incubations the cells were treated with trypsin-EDTA (0.05% Trypsin, Gibco) (40 μL /well) for 5 min at room temperature. This step removed cells from the bottom of the wells and non-ingested beads from cell surfaces. Cells were then suspended in 850 μL of incomplete media to inactivate the Trypsin. Separation of the non-ingested beads from the suspension was achieved by centrifugation (100 x *g*, 15 min, 4°C) over a cushion of 3% BSA in PBS supplemented with 4.5% D-glucose. Centrifugation of the cells formed a pellet while the free beads remained in suspension and could then be decanted off. The cell pellet was then re-suspended in incomplete medium and analyzed on a FACS Calibur flow cytometer (Becton/Dickinson).

Cells cultures were analyzed using the flow cytometer based on their fluorescence intensity. Discrete peaks representing cells containing 1, 2 or 3 beads were identified. Cells containing greater than 3 beads could also be considered as a population. By gating the regions containing 1-3 beads or >3 beads separately we were able to observe not only an overall increase in phagocytosis but also an increase in the level of phagocytic activity as represented by a greater proportion of cells ingesting more than 4 beads. The following treatments were tested: complete media, MAF (1:4), and rTNFα (2, 9, 38, and150 ng/well).

Statistical analysis was conducted by an ANOVA. Percentage values were arcsine transformed. Difference in means between treatments was determined by Tukey's test.

3.12 Nitric oxide response of goldfish macrophages to recombinant TNFα

Macrophages were isolated from goldfish kidneys and cultured until day 6 (section 2.3.). Cultures from 8 fish were spun down at 200 x *g* for 10 min and re-suspended in 1 mL of complete media. In a 96 well plate cells 50 000 cells/well from each fish were exposed to one of 10 different treatments. The treatments were as follows: cells alone, LPS (1 μg/mL), MAF (1:4) and LPS (1 μg/mL), *Aeromonas* 2 μL, rTNFα (62, 125, 250, and 500 ng/mL), rTNFα (500 ng/mL) and LPS (1 μg/mL), rTNFα without LPS removed (500 ng/mL). The final volume of the wells was 100 μL. The cells were then incubated at 20°C for 72 hours before the production of nitrite was measured using the Griess reaction assay. Seventy five µL of cell supernatants from each well was transferred to a new 96 well plate. To each well 100 µL of 1% sulfanilamide and 100 µL of 0.1% N-naphthyl-ethylenediamine was added and the plates incubated for 2 min at 20°C before the absorbance was determined at 540 nm using a microplate reader (Biotek). To determine the nitrite concentrations the absorbance values were compared to a standard curve developed from known concentrations of nitrite. Statistical analysis was conducted by an ANOVA.

3.13 Expression analysis of goldfish TNFα by RT-PCR

3.13.1 Expression of goldfish TNFα mRNA in various tissues

RNA was isolated from goldfish kidney, gill, brain and muscle tissues using TRIzol RNA extraction reagent (Invitrogen) as per the manufactures directions. First-strand synthesis of cDNA was done using 5 µg total RNA, random hexamers (Invitrogen,) and SuperScript™II RT (Invitrogen) according to manufacturer's protocols.

In order to better visualize differences in mRNA expression samples were amplified over a range of cycles (28-31). Amplification was performed using the following cycling parameters: 94 °C for 3 min; 27-31 cycles of 94 °C for 35 s; 56 °C for 40 s; 72 °C for 2 min 30 s. Once removed samples were immediately placed on ice. Primers were designed against both the TNFα-1 and 2 sequences in order to distinguish between the expression of these two isoforms. The primer sequences are reported in Table 3.2. PCR products were ran on a 1% agrose gel and stained with ethidium bromide.

3.13.2 Expression of goldfish TNFα in cultured macrophages

As is described in section 2.3 whole macrophage cultures or sorted macrophage subpopulations could be used as a source of template for mRNA expression analysis. Initial studies sought to compare the expression of TNFα1 and 2 within sorted progenitors, monocytes and mature macrophages. Sorted cells from six fish were pooled in order to obtain RNA from each subpopulation. Also included in this analysis were activated whole macrophage cultures (activation was achieved by stimulation with MAF and LPS in combination). The PCR conditions and primers were the same as those describe above in section 2.12.0 with the exception that amplification was only done for 30 cycles.

In a further experiment sorted mature macrophages from 3 fish where stimulated with either complete medium (negative control) or rTNFα-2 (500ng/mL) for 12 hr. The cells for each treatment were subsequently pooled and the RNA isolated. Expression of mRNA message for several genes including TNFα-1, TNFα-2, IL-1β, and M17 were then examined using RT-PCR. The PCR conditions for TNFα-1 and 2 were again as described previously in section 2.12.0. For goldfish IL1-β, products were amplified at 56°C for 30 cycles. Goldfish M17 was amplified at 56°C for 34 cycles. The primers used are reported in Table 3.2.

3.14 Effect of *Trypanosoma danilewskyi* stimulation on the activation of goldfish macrophages and the expression of goldfish TNFα-2

To investigate the possible response of goldfish macrophages to stimulation by *Trypanosoma danilewskyi* parasites cultured macrophages were co-incubated with *in vitro* cultured trypanosomes. Approximately 2.5 x 10^6 cells were incubated with trypanosomes at a ratio of 2:1 parasites to cells for 72 hr. Control treatments included cells alone, cells treated for activation (MAF and LPS stimulated) or parasites alone. After 72 hr a sample of the supernatants was collected and analyzed for nitrite production using the Griess reaction described in section 2.10. Statistical analysis was conducted by an analysis of variance (ANOVA). Difference in means between treatments was determined by Tukey's test. As well, the cells from each treatment were collected and their RNA extracted for RT-PCR analysis.

To measure the potential effect of stimulation by *Trypanosoma danilewskyi* on the expression of TNFα-2 the same PCR conditions and primers were used for TNFα-2 and β-actin as describe in section 2.12.0. As a type of control to confirm the presence/absences of Trypanosomes in a given treatment primers against a parasite specific product, Trans Sialidase, were also tested against each sample. The PCR conditions for these primers were as follows: 94 °C for 3 min; 35 cycles of 94 °C for 35 s; 60 °C for 40 s; 72 °C for 2 min 30 s. The Trans Sialidase specific primers are reported in Table 3.2.

Trypanosoma danilewskyi parasites were originally isolated from crucian carp but have subsequently been passage through goldfish several times. The parasites used in this study were maintained *in vitro*. Briefly, the parasites are grown in an artificial media (TDL-15) supplemented with heat inactivated goldfish serum at 20 degrees. 7 days after the beginning of a fresh culture the parasites were isolated for the experiments. The precise culture methods used were developed in our laboratory and have been described in detail previously [135].

	Forward	Reverse
prominin	5′-TGCCTCTAATTGGCCTGTTCTT-3	5′-GGCCCCCGCAGTTACC-3′
β-actin	5’-GCACGCGACTGACACTGAAG-3’	5’-GAAGGCCGCTCCGAGGTA-3

Table 3.1 Real time PCR expression primers

	Forward	Reverse
TNFα-1	**5'-GCGGGAAGGGAGATCATCATT-3'**	**5'-CCGCCTGAAGTGAAAGCCTGGTCCT-3'**
TNFα-2	**5'-TGCATATGACCCTGACGTGTG-3'**	**5'-GCCGCTCCGAGGTAAATGGTG-3'**
β-actin	**5'-TCGCTGCCCTGGTCGTTGATA-3'**	**5'-GGCGGCGGTTCCCATCTC-3'**
prominin	**5′-CTGCGTGGCCATTGGGATCCTGTAC-3′**	**5′-GCTGCAGGCCCACAACGACCC-3**
Trans Sial	**5'-CGGCGGAAATGGAAGGAATGC-3'**	**5'-CGCCGACGTTCTCACCC-3'**

Table 3.2 RT-PCR expression primers

CHAPTER 4

CHARACTERIZATION OF TUMOR NECROSIS FACTOR ALPHA OF GOLDFISH

4.1 Introduction

Tumor necrosis factor alpha (TNFα), is a highly pleiotropic pro-inflammatory cytokine that up-regulates the production of other inflammatory cytokines such as IL-1β and enhances a variety of cellular responses including phagocytosis, and chemotaxis. TNFα is produced as a type II trans-membrane protein, but it may also be cleaved by a metalloproteinase and released as a soluble peptide. Both of these forms have biologically activity [4, 137].

TNFα has been identified in different fish species including Japanese flounder [91], trout [92], carp [82, 93], catfish [94], seabream [95, 96] and turbot [97]. In some fish species such as carp and trout there are multiple isoforms of TNFα. These isoforms share a high degree of sequence identity and have similar biological activity. However, evidence suggests that the isoforms have different expression patterns at both the constitutive level and following stimulation [82, 93]. Fish TNFα has been shown to have relatively high constitutive expression in the tissues of healthy fish [93-94, 96]. However, like in mammals, an increase in TNFα mRNA message was also observed following stimulation of fish leukocytes with LPS or with mitogens such as PMA [91, 99]. Recombinant fish TNFα has been shown to enhance the expression of IL-1β, IL-8 and TNFα [98]. In carp, the addition of the protozoan parasite *Trypanoplasma borreli* to cultures of head kidney phagocytes induced an increase in expression of TNFα. It was also suggested that the presence of a TNFα-2 polymorphism correlated with enhanced resistance to the parasite due to reduced nitric oxide production [82].

The trout TNFα homolog has been shown to enhance leukocyte migration and phagocytosis [98]. Similarly, the recombinant TNFα from the gilthead seabream has been shown to increase phagocyte mobilization as well as prime their respiratory burst response [95]. In contrast, turbot recombinant TNFα failed to trigger a respiratory burst response but did have a significant effect on nitric oxide production after 72 hr of incubation [97] while the addition of pentoxifylline (PTX), a TNFα inhibitor, abrogated the nitric oxide response of carp phagocytes to LPS [82], suggesting a pivotal role for TNFα in the regulation of fish antimicrobial responses.

In this chapter, I report on the identification and molecular characterization of goldfish TNFα-1 and TNFα-2, as well as functional analyses of TNFα-2 in the goldfish.

4.2 Results

4.2.1 Sequence analysis

The complete open reading frame of goldfish TNFα-2 (Figure 4.1) and a partial sequence of goldfish TNFα-1 were obtained. A BLASTx search of goldfish TNFα-2 against the NCBI Genbank database identified a carp TNFα-2 sequence (accession number AJ311801) as having the highest blast score (E value of 7×10^{-99}). Likewise goldfish TNFα-1 shared the greatest similarity with carp TNFα-1 (accession number CAC84641, E value of 1×10^{-69}). Alignment of the goldfish TNFα-1 and 2 nucleotide sequences indicated the high degree of conservation between these two sequences which had an 83% identity (Figure 4.2).

The open reading for goldfish TNFα-2 is 687 base pairs in length and encodes for a 228 amino acid protein with a predicted size of approximately 25 kD (Figure 4.1). The translated goldfish sequence contained all the basic elements of the TNF genes already identified in fish and other vertebrates. They included a trans-membrane domain and metalloproteinase cleavage site (for release of the mature protein) as well as the TNF family signature and two conserved cysteine residues that are known to influence TNFα tertiary structure (Figure 4.4). An amino acid alignment of the predicted goldfish TNFα-2 protein with other sequences from fish and mice shows a the high degree of overall conservation between the goldfish and carp sequences as well as the conservation of the previously discussed features across all of the sequences examined (Figure 4.4). The amino acid identity of goldfish TNFα-2 with carp TNFα-2 and 1 were 87% and 75%, respectively. The identity with other fish sequences ranged between 36-54% and was significantly lower for murine TNFα (28% identity) (Figure 4.4). An amino acid alignment of the goldfish TNFα-1 partial sequence was also done (Figure 4.3).

Phylogenetic analysis of both goldfish TNFα isoforms with TNFα sequences from other fish species as well as those for mammals confirmed the close relationship of the goldfish and carp TNFα isoforms (Figure 4.5). Zebrafish which are also cyprinid fish, grouped with goldfish and carp. Trout TNFα, sequences which also occur in multiple isoforms, group separately from goldfish and carp (Figure 4.5). This suggests that the isoforms seen in trout arose separately from those of carp and goldfish.

4.2.2 Production of recombinant goldfish TNFα-2 using a prokaryotic expression systems

A PCR product encoding the majority of the cleaved mature TNFα protein was cloned into the pET SUMO expression vector (Invitrogen) for transfection into *E. coli* (Figure 4.6). IPTG induction trials found that the majority of the recombinant protein was sequestered in the insoluble fraction (inclusion bodies), and that maximum induction occurred after 4 hr (Figure 4.7). Recombinant goldfish TNFα-2 was isolated under denaturing conditions (8M urea) using MagneHis Ni-Particles (Promega). The protein was eluted using 500 mM imidizole and its presence was confirmed by western blot using anti-His antibody (Figure 4.8). To promote re-naturation the purified protein was incubated at 4°C overnight in 40 mM Tris.

4.2.3 Expression of TNFα in goldfish tissues and cultured macrophages

Specific primers were designed against the most divergent portions of both the TNFα-1 and 2 sequences in order to examine the differential expression of these two forms. Initially, it was my goal to conduct this analysis through real time PCR. However, I was unable to identify working primers that would meet the efficiency requirements of the validation experiment. Therefore, the analyses were done using semi-quantitative RT-PCR, that was optimized for qualitative comparison. The expression of goldfish TNFα was examine in two important immune relevant tissues (the kidney and gill) as well as in the brain and muscle. Goldfish TNFα-1 was expressed constitutively in both the kidney and gill while TNFα-2 was only significantly expressed in the gills. Neither isoform was significantly expressed in the brain or muscle, however, very low expression levels of TNFα-1 were observed (Figure 4.9).

As the primary goal of this study was to functionally characterize goldfish TNFα in cultured goldfish macrophages it was particularly important for us to examine mRNA expression in *in vitro*-derived primary macrophages. The examination of TNFα-1 and TNFα-2 in sorted subpopulations of progenitors, monocytes and mature macrophages yielded an interesting pattern of differential expression. While both TNFα-1 and 2 were highly expressed in activated macrophages, TNFα-2 did not appear to be expressed in non-activated mature macrophages, while TNFα-1 was expressed constitutively in these cells prior to activation. Neither isoform was significantly expressed in the early progenitor or monocyte subpopulations (Figure 4.10).

The effect of recombinant goldfish TNFα-2 on the expression of pro-inflammatory cytokine, IL-1β in the mature macrophage subpopulation was also determined. TNFα-1 was highly expressed in both non-activated and activated macrophages. Both TNFα-2 and IL1-β appeared to be up-regulated following treatment of macrophages with recombinant TNFα-2 for 12 hrs (Figure 4.11). Note that there was some expression of TNFα-2 in non-activated cell subpopulation, which is in contrast to the previous observation (see Figure 4.10). These results suggest that sorting of macrophages by FACS may activate them resulting in up regulation of genes such as TNFα-2.

4.2.4 Effect of Trypanosoma danilewskyi stimulation on the activation of goldfish macrophages and the expression of goldfish TNFα-2

In order to examine the potential response of goldfish macrophages to the blood born protozoan *Trypanosoma danilewskyi* the macrophages were co-incubated with cultured parasites. Macrophages co-incubated for 72 hr with *T. danilewskyi* did not express TNFα-2, while cells stimulated with mitogen activated cell supernatants (MAF) and bacterial endotoxin (LPS) did. Non-stimulated macrophages did not express TNFα-2 (Figure 4.12A).

To assess whether goldfish macrophages were activated, I measured their ability to mount a nitric oxide response. Trypanosome stimulated macrophages exhibited a nitric oxide response higher than that of non-activated macrophages, but this response was not significantly different. Only MAF and LPS was significantly higher than the negetive control (df=2, F value=8.98, $p<0.0157$) (Figure 4.12B). These results suggest that the production of nitric oxide and the expression of TNFα-2 by activated macrophages may not be related.

4.2.5 Effect of recombinant goldfish TNFα-2 on chemotaxis of goldfish macrophages

Recombinant goldfish TNFα-2 induced a chemotactic response of goldfish macrophages in a dose dependent manner (df=8, F value=19.75, $p<0.0001$) (Figure 4.13). The highest chemotactic response was observed at a recombinant TNFα-2 concentration of 10 ng/well or approximately 67 ng/mL. This response was 4 times higher compared to that of the negative control (medium alone), and similar to that induced by LPS (positive control). As expected the chemotactic response decreased with the increasing doses of the TNFa-2, indicating the macrophages were responding to a concentration gradient (Figure 4.13).

In order to account for the random chemokinetic activity induced by TNFα-2 and LPS, equal concentrations (10 ng/well and 5 ng/well, respectively) of the chemo-attractive agents were placed into both the upper and lower wells of the migration apparatus in certain experimental groups. The numbers of macrophages observed to have crossed the separating membrane for these treatments was not significantly different from that of the medium alone control (Figure 4.14).

4.2.6 Effect of recombinant goldfish TNFα-2 on the phagocytic response of goldfish macrophages

Using a flow cytometry based assay developed from methods described by Li *et. al.* [32], I measured the degree to which macrophages were ingesting florescent latex beads. Based on the fluorescence intensity, the flow cytometer histogram peaks co-responded to macrophages that had ingested 1, 2, 3 or more than 3 fluorescent latex beads (Figure 4.15A). Following activation of macrophages with MAF or recombinant TNFα-2, I observed both an increase in the numbers of cells with ingested beads (Figure 4.16) as well as an increase in the number of ingested beads per macrophage indicating that TNFα-2 enhance phagocytic activity of goldfish macrophages (Figure 4.15B). The number of TNFα-2-activated macrophages that ingested 3 or more latex beads was related to the amount of TNFα-2 added to the cultures, and was significantly higher compared to the non-activated cells (Figure 4.17). Statistical analysis indicated that treatment of macrophages with TNFα-2 augmented the capacity of activated macrophages to ingest latex beads (df=5, F value=20.18, p=<.0001). The analysis of the data using Tukey's test indicated that experimental groups where activation molecules were added had significantly greater percentage of phagocytic cells in the cultures. The induction of phagocytosis was dose-dependent such that the highest concentrations of TNFα-2 (370-1500 ng/mL) or MAF induced significantly higher phagocytic activity compared to the lower amounts of TNFα-2 (23 ng/mL) (Figure 4.17).

4.2.7 Effect of recombinant goldfish TNFα-2 on the nitric oxide response of goldfish macrophages

Recombinant goldfish TNFα-2 induced a nitric oxide response 72 hr after stimulation in a dose dependent manner (df=9, F value=3.03, p=0.0075) (Figure 4.18). The highest concentration of TNFα-2 tested (500 ng/mL) induced the highest nitrite production, a mean of 9.1 ± 1.6 μM nitrite (per 5 X 10^{-4} cells/72 hrs). This was almost 2.5 times the nitrite production observed for the non-stimulated or LPS alone experimental groups and similar to nitrite production induced by MAF & LPS or *Aeromonas* (Figure 3.18). The synergistic effect observed for nitrite production of macrophages following treatment with MAF & LPS, was not observed when TNFα-2 and LPS were added to the cultures concurrently. The absence of the synergistic effect was not due to the contaminating LPS in the recombinant TNFa-2 preparations, since the removal of LPS or the add-back of 1μg/mL of LPS to the preparations did not induce a greater response compared to LPS alone (Figure 4.18).

4.3 Discussion

Two isoforms of TNFα have been identified in the goldfish. Goldfish TNF gene sequences were similar to those of carp TNFα-1 and TNFα-2. Consequently, I have named these genes as goldfish TNFα-1 and TNFα-2 based on their similarity to the carp TNFα-1 and TNFα-2 isoforms. The complete open reading frame of goldfish TNFα-2 was identified and the predicted amino acid sequence shared hallmark features with other previously described fish sequences [91-95, 97]. These include a trans-membrane region, cleavage site, TNF family signature and two conserved cysteine residues.

The constitutive tissue expression of TNFα in different fishes has been shown to be highly variable. When the first TNF gene was identified in the Japanese flounder, little or no constitutive tissue expression was reported [91]. However, TNFα subsequently identified in catfish, seabream and carp, all appeared to be expressed in a number of tissues [93-94, 96]. In carp, a broad pattern of tissue expression was attributed to only one isoform (TNFα-3), while the other isoforms (TNFα-1 and 2) were only identifiable in the gill [93]. Goldfish TNFα-1 and 2 which appear to be homologous to carp are also predominantly expressed in the gills, however, goldfish TNFα-1 also exhibits constitutive expression in the kidney as well as in the brain and muscle (levels in the later two tissues were low).

From mammalian studies, we know that the primary producers of TNFα are the macrophages [137], similarly, fish macrophages have been shown to produce TNFα [92, 94, 98]. Distinct profiles of mammalian TNFα isoforms have been shown to be differentially up-regulated depending on the stimulating factor (ie. LPS or GM-CSF) [87]. The mammalian isoforms are distinct from those reported in fish, since they are generated by post-transcriptional/translational modifications rather than being encoded by different genes [87, 92-93].

Goldfish TNFα-1 and TNFα-2 isoforms were differentially expressed in goldfish macrophages. TNFα-1 was constitutively expressed in non-activated mature macrophages and appeared to be further up-regulated in activated macrophage cultures. Goldfish TNFα-2, in contrast, was only up-regulated upon activation. In the carp, a differential pattern of expression of TNFα isoforms was reported, where one isoform was expressed in both non-activated and activated macrophages, while the other was expressed only upon activation of the cells [82]. Unlike the goldfish TNFα isoform expression, it was the TNFα-2 isoform that was constitutively expressed in carp. In the trout, multiple TNFα isoforms are also shown to exhibit differential expression in stimulated macrophages [92]. The type 1 isoform in trout was only expressed at very low levels in stimulated trout macrophages compared to the type 2 [92]. In addition, the recombinant trout TNFα-1 did not up-regulate native trout TNFα-1, while recombinant TNFα-2 induced increased expression of both native TNFα isoforms [98]. We also found that the recombinant goldfish TNFα-2 induced the expression of native TNFα, which was dependent on the activation status of macrophages. Although no clear functional differences have been observed for different isoforms of fish TNFα, the patterns of expression observed for the TNFα isoforms of goldfish and other fish suggest a potentially distinct immune regulatory roles of different TNFα isoforms.

It has been reported that the protozoan parasite *Trypanoplasma borreli* induced the expression of TNFα in head kidney phagocytes [82]. In the same study, Saeji et al. [82] reported that nitric oxide production was critical for the regulation of the susceptibility of carp to *T. borreli*, since a decrease in the nitric oxide response was related to greater resistance of carp to the parasite. We examined whether a related parasite *T. danilewskyi,* which infects both carp and goldfish, could induce TNFα expression in cultured goldfish macrophages. We found that co-cultivation of goldfish macrophages with the parasites for up to 72 hrs, did not result in increased TNFα expression. Furthermore, T. danilewskyi induced a modest increase in the nitrite production by activated macrophages which was notably lower than that induced by MAF & LPS. These results suggest that unlike *T.boreli,* T. danilewskyi does not have the ability to significantly activate goldfish macrophages.

Because TNFα as a pleiotropic cytokine that participates in the regulation of the innate immune responses, we generated a functional recombinant molecule and examined the ability of this molecule to influence macrophage functions known to be important for proper innate immune response. We knew that functional studies using the trout recombinant TNFα have demonstrated the ability of this cytokine to induce a chemotactic response of macrophages [98]. Our results confirm the earlier observations in that recombinant goldfish TNFα also induced the chemotactic response of mature goldfish macrophage in a dose-dependent manner. The concentration of TNFα required to induce maximum chemotactic response was approximately 67 ng/mL, while higher amounts of TNFα inhibited the chemotactic response of cultured goldfish macrophages.

It should be noted that the concentration of recombinant TNFα required to induce a chemotactic response of goldfish macrophages was significantly higher than physiologically relevant concentrations that would induce a similar effect *in vivo*. The production of the recombinant TNFα using a prokaryotic expression system and the purification of the molecule under denaturing conditions probably meant that a large fraction of the measurable protein content was not properly folded.

The ability of recombinant trout TNFα to mediate phagocytosis has been reported. [98], as was the induction of the respiratory burst response of both seabream and turbot phagocytes [95, 97]. We investigated the ability of goldfish recombinant TNFα to induce the phagocytosis of goldfish macrophages using flow cytometry which allowed us to examine this inflammatory response of macrophages at a population as well as individual cell level. Under the influence of goldfish TNFα–2 stimulation, not only did a greater proportion of macrophages phagocytize fluorescent latex beads but also a dose dependent increase in the number of latex beads per macrophage was observed. The induction of phagocytic response in goldfish macrophages by recombinant TNFα-2 was the highest when the cells were treated with greater than 370ng/mL of the recombinant protein.

Recombinant goldfish TNFα-2 induced a potent antimicrobial response of activated macrophages, the production of reactive nitrogen intermediates. A does of 500 ng/mL of goldfish TNFα induced a nitric oxide response that was equal to or greater than that induced by exposure of macrophages to *Aeromonas*. It has been shown that LPS in combination with MAF synergize to induce a maximal nitric oxide response in goldfish macrophages [138]. The synergistic effect of LPS and TNFa was not observed in our experiments.

Teleost TNFα, like the mammalian cytokine appear to be a potent and pleiotropic regulator of the innate immune response. In this study, goldfish TNFα produced using prokaryotic expression system was shown to be biologically active since it induced dose-dependent pro-inflammatory responses: chemotaxis, phagocytosis, and production of reactive nitrogen intermediates, which are the corner stone functions of fish innate immunity.

```
    atg atg ata gat gtt gag agt cag tta gct gaa
  1 --- --- --- +-- --- --- -+- --- --- --+ ---
    tac tac tat cta caa ctc tca gtc aat cga ctt
    Met Met Ile Asp Val Glu Ser Gln Leu Ala Glu

    gaa gga gcg cag gtg acg gtg tcg agg agg agg
 34 --- --- +-- --- --- -+- --- --- --+ --- ---
    ctt cct cgc gtc cac tgc cac agc tcc tcc tcc
    Glu Gly Ala Gln Val Thr Val Ser Arg Arg Arg

    tcc ggt gtc tgg cgg gtg tgt ggg gtc ctg ctg
 67 --- +-- --- --- -+- --- --- --+ --- --- ---
    agg cca cag acc gcc cac aca ccc cag gac gac
    Ser Gly Val Trp Arg Val Cys Gly Val Leu Leu

    gct gtg gcc ctg tgt gcc gcc gct gct gtc tgc
100 +-- --- --- -+- --- --- --+ --- --- --- +--
    cga cac cgg gac aca cgg cgg cga cga cag acg
    Ala Val Ala Leu Cys Ala Ala Ala Ala Val Cys

    ttc aca ttc aac aag tct cag aac aat cag gaa
133 --- --- -+- --- --- --+ --- --- --- +-- ---
    aag tgt aag ttg ttc aga gtc ttg tta gtc ctt
    Phe Thr Phe Asn Lys Ser Gln Asn Asn Gln Glu

    agt gga aat gag ctg agg ctc aca tta aga gac
166 --- -+- --- --- --+ --- --- --- +-- --- ---
    tca cct tta ctc gac tcc gag tgt aat tct ctg
    Ser Gly Asn Glu Leu Arg Leu Thr Leu Arg Asp

    cat ctt tca aaa gaa aat gtc act tcc aag gcc
199 -+- --- --- --+ --- --- --- +-- --- --- -+-
    gta gaa agt ttt ctt tta cag tga agg ttc cgg
    His Leu Ser Lys Glu Asn Val Thr Ser Lys Ala

    gcg atc cat tta aca ggt gca tat gac cct gac
232 --- --- --+ --- --- --- +-- --- --- -+- ---
    cgc tag gta aat tgt cca cgt ata ctg gga ctg
    Ala Ile His Leu Thr Gly Ala Tyr Asp Pro Asp
    gtg tgt acg gac aac cta gac tgg aaa cag aac
    --- --+ --- --- --- +-- --- --- -+- --- ---
265 cac aca tgc ctg ttg gat ctg acc ttt gtc ttg
    Val Cys Thr Asp Asn Leu Asp Trp Lys Gln Asn

    cag gac cag gct ttt gtt tca ggt ggc ttg aaa
298 --+ --- --- --- +-- --- --- -+- --- --- --+
    gtc ctg gtc cga aaa caa agt cca ccg aac ttt
    Gln Asp Gln Ala Phe Val Ser Gly Gly Leu Lys

    cta gtg gac aga gag atc atc att cct tac gac
331 --- --- --- +-- --- --- -+- --- --- --+ ---
    gat cac ctg tct ctc tag tag taa gga atg ctg
    Leu Val Asp Arg Glu Ile Ile Ile Pro Tyr Asp

    ggc att tac ttc gtc tac agt cag gtg tct ttc
364 --- --- +-- --- --- -+- --- --- --+ --- ---
    ccg taa atg aag cag atg tca gtc cac aga aag
    Gly Ile Tyr Phe Val Tyr Ser Gln Val Ser Phe

    cac atc agc tgc aag gct gac gtg act gag gaa
397 --- +-- --- --- -+- --- --- --+ --- --- ---
    gtg tag tcg acg ttc cga ctg cac tga ctc ctt
    His Ile Ser Cys Lys Ala Asp Val Thr Glu Glu

    cac gag ggc gtg cac atg agc cac gca gtg ttg
430 +-- --- --- -+- --- --- --+ --- --- --- +--
    gtg ctc ccg cac gtg tac tcg gtg cgt cac aac
    His Glu Gly Val His Met Ser His Ala Val Leu

    cgc ttc tcg gag tcc tac gcc agc tac aag ccg
463 --- --- -+- --- --- --+ --- --- --- +-- ---
    gcg aag agc ctc agg atg cgg tcg atg ttc ggc
    Arg Phe Ser Glu Ser Tyr Ala Ser Tyr Lys Pro

    ctc ttc agc gcg atc cgc tcg gcc tgc gtg cac
496 --- -+- --- --- --+ --- --- --- +-- --- ---
    gag aag tcg cgc tag gcg agc cgg acg cac gtg
    Leu Phe Ser Ala Ile Arg Ser Ala Cys Val His
    gcg act gac act gaa gac ctg tgg tac aac acc
529 -+- --- --- --+ --- --- --- +-- --- --- -+-
    cgc tga ctg tga ctt ctg gac acc atg ttg tgg
    Ala Thr Asp Thr Glu Asp Leu Trp Tyr Asn Thr

    att tac ctc gga gcg gcc ttc aac ctg cga gct
562 --- --- --+ --- --- --- +-- --- --- -+- ---
    taa atg gag cct cgc cgg aag ttg gac gct cga
    Ile Tyr Leu Gly Ala Ala Phe Asn Leu Arg Ala

    gga gac aaa ctg cgc acc gac acc acc aca gaa
495 --- --+ --- --- --- +-- --- --- -+- --- ---
    cct ctg ttt gac gcg tgg ctg tgg tgg tgt ctt
    Gly Asp Lys Leu Arg Thr Asp Thr Thr Thr Glu

    ctc ctg ccc cgc gtg gaa agc gaa aac gga aag
628 --+ --- --- --- +-- --- --- -+- --- --- --+
    gag gac ggg gcg cac ctt tcg ctt ttg cct ttc
    Leu Leu Pro Arg Val Glu Ser Glu Asn Gly Lys

    acc ttc ttc ggg gtg ttt gct tta tga
661 --- --- --- +-- --- --- -+- --- ---
    tgg aag aag ccc cac aaa cga aat act
    Thr Phe Phe Gly Val Phe Ala Leu ***
```

Figure 4.1 Goldfish TNFα-2 ORF nucleotide and amino acid sequence 5’ to 3’.

```
GFTNF2 GGACATAGCTGAACTTACTAACAGACACAATGATGATAGATGTTGAGAGTCAGTTAGCTG 60
GFTNF1 ------------------------------------------------------------


GFTNF2 AAGAAGGAGCGCAGGTGACGGTGTCGAGGAGGAGGTCCGGTGTCTGGCGGGTGTGTGGGG 120
GFTNF1 ------------------------------------------------------------


GFTNF2 TCCTGCTGGCTGTGGCCCTGTGTGCCGCCGCTGCTGTCTGCTTCACATTCAACAAGTCTC 180
GFTNF1 ------------------------------------------------------------


GFTNF2 AGAACAATCAGGAAAGTGGAAATGAGCTGAGGCTCACATTAAGAGACCATCTTTCAAAAG 240
GFTNF1 --------------------------------------TTAAGAGACCATCTTTCAAAAG 22
                                             **********************

GFTNF2 AAAATGTCACTTCCAAGGCCGCGATCCATTTAACAGGTGCATATGACCCTGACGTGTGTA 300
GFTNF1 CAAACGTCACTTCCAAGGCTGCCATCCATTTAACAGGTGCGTATGAACCTAAAGTGTCCA 82
        *** ************** ** ***************** ***** *** * ****  *

GFTNF2 CGGACAACCTAGACTGGAAACAGAACCAGGACCAGGCTTTTGTTTCAGGTGGCTTGAAAC 360
GFTNF1 AAGACACCCTTTACTGGAGAAAGGACCAGGACCAGGCTTTCACTTCAGGCGGCTTGAAAT 142
         **** ***  ****** * ** ****************   ****** *********

GFTNF2 TAGTGGACAGAGAGATCATCATTCCTTACGACGGCATTTACTTCGTCTACAGTCAGGTGT 420
GFTNF1 TAGCGGGAAGGGAGATCATCATTCCTACGGATGGCATTTACTTTGTCTACAGTCAGGTGT 202
       *** **  ** ***************   ** *********** ****************

GFTNF2 CTTTCCACATCAGCTGCAAGGCTGACGTGACTGAGGAACACGAGGGCGTGCACATGAGCC 480
GFTNF1 CTTTCCACATCAGATGCAAGACTGACATTCCTGAGGACCACGATGTTGTGCAAATGAGCC 262
       ************* ****** ***** *  ******* ***** *  ***** *******

GFTNF2 ACGCAGTGTTGCGCTTCTCGGAGTCCTACGCCAGCTACAAGCCGCTCTTCAGCGCGATCC 540
GFTNF1 ACATAGTGTTCCGCTACTCTGATTCCTATGGCAGCTACAAGCCACTTTTCAGCGCAATCC 322
       **  ****** **** *** ** ***** * ************ ** ******** ****

GFTNF2 GCTCGGCCTGCGTGCACGCGACTGACACTGAAGACCTGTGGTACAACACCATTTACCTCG 600
GFTNF1 GCTCGGCCTGCGAGCAGGCGACAGACTCTGACGATCTGTGGTACAACACGATTTATGTCG 382
       ************ *** ***** *** **** ** ************** *****  ***

GFTNF2 GAGCGGCCTTCAACCTGCGAGCTGGAGACAAACTGCGCACCGACACCACCACAGAACTCC 660
GFTNF1 GTGCGGCCTTCAGCCTGCGAGCCGAAGACAGGCTGTGCACCAATACGACTATAGCACTCC 442
       * ********** ********* * *****  *** ***** * ** ** * ** *****

GFTNF2 TGCCCCGCGTGGAAAGCGAAAACGGAAAGACCTTCTTCGGGGTGTTTGCTTTATGA 716
GFTNF1 TGCCTCGCGTCGAAAGCGACAACGGAAAGACCTTCTTCGGGGTGTTTC-------- 490
       **** ***** ******** ***************************

Sequence identity 83%
```

Figure 4.2 Nucleotide alignment of goldfish TNFα-1 and 2 isoform sequences. Complete conservation of sequence is indicated by asterisks (*). Percent identity is reported as 83%.

```
goldfishTNF1 ------------------------------------------------------------
carpTNF1     MMDLESQLLEEGGL----------LPLPQVMVSRRKSGSSKSGVWRVCGVLLAVALCAAA 50
carpTNF2     MMDLENQFLEEG-A----------LPLPQVMVSRRKSG-----VWRVCGVLLAVALCAAA 44
catfishTNF   -MASDSQVVLDVD-------------GPRVTIVREKASWSSSGVWRTCGVLLAVALCAAA 46
seabreamTNF  -MGAYTTAPCDLEM---------GPEERTVV--LIEKKSSTGWMWKVSVALLIAALCFAG 48
flounderTNF  -------------------------------------------MCKVLGGLFIVALCLGG 17
troutTNF2    -MEGYAMTPEDMERGLENSLVDSGPVYKTTVTAVAERKASRGWLWRLCGVLLIAALCAAA 59
troutTNF1    -MEGYAMTPEDMER---------GPVYNTTVTAVAEGKASRGWLWRLCGVLLIAGLCAAA 50
mouseTNF     --MSTESMIRDVEL--------------AEEALPQKMGGFQNSRRCLCLSLFSFLLVAGA 44

goldfishTNF1 ----------------------------------LRDH-SKANVTSKAAIHLTGAYEPKV 25
carpTNF1     AVCFTLNKSQNNQEGGN------------ALRLTLRDHLSKANVTSKAAIHLIGAYEPKV 98
carpTNF2     AVCFTLNKSQNNQEGGN------------ALRLTLRDHLSKENVTSKVAIHLTGAYDPDV 92
catfishTNF   AVCFSQNKTHNKPDE----------------TQEIKHSLRQISQTAKAAIHLSGHYNPQV 90
seabreamTNF  VLLFAWYWNGKPEILIHSGQSEALTKKDHAEKTDPHSTLKRISSKAKAAIHLEGSYDEDE 108
flounderTNF  VLAFSWYTN-KSEMMTQSGQTAALSQKDCAEKTEPHNTLRQISSRAKAAIHLEGRDEEDE 76
troutTNF2    ALLFAWCQHGRLATMQDGMEP---QLEIFIGAKDTHNTLKQIAGNAKAAIHLEGEYNPNL 116
troutTNF1    ALLFAWCQHGRPSTMQDEIEP---QLEILIGAKDTHHTLKQIAGNAKAAIHLEGEYNPNL 107
mouseTNF     TTLFCLLNFGVIGPQRDEKFP-----NGLPLISSMAQTLTLRSSSQNSSDKPVAHVVANH 99
                                                           : : :  .    .

goldfishTNF1 --SKDTLYWRKDQDQAFTSGGLKLAGREIIIPTDGIYFVYSQVSFHIRCKT-------DI 76
carpTNF1     --STETLDWKKNQDQAFTSGGLKLVEREIIIPTDGIYFVYSQVSFHINCKT-------NM 149
carpTNF2     --CKDNLDWKQNQDQAFVSGGLELVDREIIIPNDGIYFVYSQVSFHISCKH-------DM 143
catfishTNF   --SSVSMQWFDNADQSFSSG-LKLEDNEIKILRDGLYFVYSQASYRLLCKA-------EG 140
seabreamTNF  -GLKDQVEWKNGQGQAFAQGGFRLVDNKIVIPHTGLYFVYSQASFRVSCS--DGDE--EG 163
flounderTNF  ETSENKLVWKNDEGLAFTQGGFELVDNHIIIPRSGLYFVYSQASFRVSCSSDDADDGKEA 136
troutTNF2    --TADTVQWRKDDGQAFSQGGFKLQGNQILIPHTGLFFVYSQASFRVKCN---------G 165
troutTNF1    --SADTVQWRKDDGQAFSQGGFELQGNQILIPHTGLFFVYSQASFRVKCN---------S 156
mouseTNF     -QVEEQLEWLSQRANALLANGMDLKDNQLVVPADGLYLVYSQVLFKGQGCP--------- 149
                   : * .    ::  . : *  ..: :   *:::****. ::

goldfishTNF1 PEDH-DVVQMSHIVFRYSDSYGSYKPLFSAIRSACEQATDS-----DDLWYNTIYVGAAF 130
carpTNF1     TEDH-DLVHMS-TVLRYSDSYGRYMPLFSAIRTACAQASNT-----DDLWYNTIYLGAAF 202
carpTNF2     TEDQ-DVVHMSHAVLRYSESYGSYKPLFSAIRSACVHASDS-----EDLWYNTIYLGAAF 197
catfishTNF   DETEGEVMHMSHKVSRWSDSYSSWKPLLSATRSACKKTTEEY----QKYWYGAVYLGAAF 196
seabreamTNF  AGRH--LTPLSHRISRYSESMGSDVSLMSAVRSACQNTAQEDSYSDGRGWYNTIYLGAVF 221
flounderTNF  AEKH--LTSISHRVWLFTESLGTQVSLMSAVRSACQ-KSQEDAYRDGQGWYNAIYLGAVF 193
troutTNF2    PGER--TTPLSHVIWRYSDSIGDKGNLLSGVRSVCQQNYGNDESNIGEGWYNAVYLSAVF 223
troutTNF1    PGEH--TTPLSHIIWRYSDSIGVNANLLSGVRSVCQQNYGDAESKIGEGWYNAVYLGAVF 214
mouseTNF     -----DYVLLTHTVSRFAISYQEKVNLLSAVKSPCPKDTPEG--AELKPWYEPIYLGGVF 202
                      ::  :  :: *      *:*. :: *              ** .:*:...*

goldfishTNF1 SLRAEDRLCTNTTIALLPRVESDNGKTFFGVF-- 162       %identity
carpTNF1     KLRAGDRLRTETTEELLPSVETGDGKTFFGVFAL 236           75
carpTNF2     NLRARDRLRTETTKELLPRVESENGKTFFGVFAL 231           75
catfishTNF   NLKAGDRLRTVMDEKLLPKVESAGGKTFFGTFSL 230           51
seabreamTNF  QLNRGDKLETETN--QLSELETDEGKTFFGVFAL 253           45
flounderTNF  QLNEGDKLWTETN--MLSELETESGKTFFGVFAL 225           42
troutTNF2    QLNEGDKLWTETN--RLTDVEPEQGKNFFGVFAL 255           43
troutTNF1    QLNEGDKLWTETN--RLTDVEPEQGKNFFGVFAL 246           44
mouseTNF     QLEKGDQLSAEVNLPKYLDFAE-SGQVYFGVIAL 235           25
                 .*.  *:* :         .    *: :**.:
```

Figure 4.3 Amino acid alignment of goldfish TNFα-1 partial sequence with sequences from carp, catfish, seabream, flounder trout and mouse. Fully conserved residues are indicated by an asterisk (*), partially conserved and semi-conserved substitutions are represented by ":" and "." respectively.

```
goldfishTNF2 MMIDVESQLAEEGA---------------QVTVSRRRSG-----VWRVCGVLLAVALCAA 40
carpTNF2     -MMDLENQFLEEGALP-----------LPQVMVSRRKSG-----VWRVCGVLLAVALCAA 43
carpTNF1     -MMDLESQLLEEGGLLP----------LPQVMVSRRKSGSSKSGVWRVCGVLLAVALCAA 49
catfishTNF   --MASDSQVVLDVDGP-------------RVTIVREKASWSSSGVWRTCGVLLAVALCAA 45
seabreamTNF  --MGAYTTAPCDLEM---------GPEERTVV--LIEKKSSTGWMWKVSVALLIAALCFA 47
flounderTNF  --------------------------------------------MCKVLGGLFIVALCLG 16
troutTNF2    --MEGYAMTPEDMERGLENSLVDSGPVYKTTVTAVAERKASRGWLWRLCGVLLIAALCAA 58
troutTNF1    --MEGYAMTPEDMER---------GPVYNTTVTAVAEGKASRGWLWRLCGVLLIAGLCAA 49
mouseTNF     --MSTESMIRDVELA---------------EEALPQKMGGFQNSRRCLCLSLFSFLLVAG 43
                                                                *:   *  .

                                              ↘
goldfishTNF2 AAVCFT---FNKSQNNQESGNE---------LRLTLRDHLSKENVTSKAAIHLTGAYDPD 88
carpTNF2     AAVCFT---LNKSQNNQEGGNA---------LRLTLRDHLSKENVTSKVAIHLTGAYDPD 91
carpTNF1     AAVCFT---LNKSQNNQEGGNA---------LRLTLRDHLSKANVTSKAAIHLIGAYEPK 97
catfishTNF   AAVCFS---QNKTHNKPDETQE---------IKHSLR-QISQ---TAKAAIHLSGHYNPQ 89
seabreamTNF  GVLLFAWYWNGKPEILIHSGQSEALTKKDHAEKTDPHSTLKRISSKAKAAIHLEGSYDED 107
flounderTNF  GVLAFSWYTN-KSEMMTQSGQTAALSQKDCAEKTEPHNTLRQISSRAKAAIHLEGRDEED 75
troutTNF2    AALLFAWCQHGRLATMQDGMEP---QLEIFIGAKDTHNTLKQIAGNAKAAIHLEGEYNPN 115
troutTNF1    AALLFAWCQHGRPSTMQDEIEP---QLEILIGAKDTHHTLKQIAGNAKAAIHLEGEYNPN 106
mouseTNF     ATTLFCLLNFGVIGPQRDEKFP-----NGLPLISSMAQTLTLRSSSQNSSDKPVAHVVAN 98
             ..  *            .                     :       : : :  .    .
                                                               ✧
goldfishTNF2 V--CTDNLDWKQNQDQAFVSGGLKLVDREIIIPYDGIYFVYSQVSFH-ISCKADV----- 140
carpTNF2     V--CKDNLDWKQNQDQAFVSGGLELVDREIIIPNDGIYFVYSQVSFH-ISCKHDM----- 143
carpTNF1     V--STETLDWKKNQDQAFTSGGLKLVEREIIIPTDGIYFVYSQVSFH-INCKTNM----- 149
catfishTNF   V--SSVSMQWFDNADQSFSSG-LKLEDNEIKILRDGLYFVYSQASYR-LLCKAEG----- 140
seabreamTNF  E-GLKDQVEWKNGQGQAFAQGGFRLVDNKIVIPHTGLYFVYSQASFR-VSCS--DGDE-- 161
flounderTNF  EETSENKLVWKNDEGLAFTQGGFELVDNHIIIPRSGLYFVYSQASFR-VSCSSDDADDGK 134
troutTNF2    L--TADTVQWRKDDGQAFSQGGFKLQGNQILIPHTGLFFVYSQASFR-VKCN-------- 164
troutTNF1    L--SADTVQWRKDDGQAFSQGGFELQGNQILIPHTGLFFVYSQASFR-VKCN-------- 155
mouseTNF     H-QVEEQLEWLSQRANALLANGMDLKDNQLVVPADGLYLVYSQVLFKGQGCP-------- 149
                    : * .    ::  . : *  ..: :   *:::****. ::   *
                                               ✧
goldfishTNF2 TEEH-EGVHMSHAVLRFSESYASYKPLFSAIRSACVHATDT-----EDLWYNTIYLGAAF 194
carpTNF2     TEDQ-DVVHMSHAVLRYSESYGSYKPLFSAIRSACVHASDS-----EDLWYNTIYLGAAF 197
carpTNF1     TEDH-DLVHMS-TVLRYSDSYGRYMPLFSAIRTACAQASNT-----DDLWYNTIYLGAAF 202
catfishTNF   DETEGEVMHMSHKVSRWSDSYSSWKPLLSATRSACKKTTEEY----QKYWYGAVYLGAAF 196
seabreamTNF  EGAGRHLTPLSHRISRYSESMGSDVSLMSAVRSACQNTAQEDSYSDGRGWYNTIYLGAVF 221
flounderTNF  EAAEKHLTSISHRVWLFTESLGTQVSLMSAVRSACQ-KSQEDAYRDGQGWYNAIYLGAVF 193
troutTNF2    -GPGERTTPLSHVIWRYSDSIGDKGNLLSGVRSVCQQNYGNDESNIGEGWYNAVYLSAVF 223
troutTNF1    -SPGEHTTPLSHIIWRYSDSIGVNANLLSGVRSVCQQNYGDAESKIGEGWYNAVYLGAVF 214
mouseTNF     -----DYVLLTHTVSRFAISYQEKVNLLSAVKSPCPKDTPEG--AELKPWYEPIYLGGVF 202
                      ::  :  :: *      *:*. :: *              ** .:**...*

goldfishTNF2 NLRAGDKLRTDTTTELLPRVESENGKTFFGVFAL 228         %identity
carpTNF2     NLRARDRLRTETTKELLPRVESENGKTFFGVFAL 231            87
carpTNF1     KLRAGDRLRTETTEELLPSVETGDGKTFFGVFAL 236            75
catfishTNF   NLKAGDRLRTVMDEKLLPKVESAGGKTFFGTFSL 230            54
seabreamTNF  QLNRGDKLETETN--QLSELETDEGKTFFGVFAL 253            39
flounderTNF  QLNEGDKLWTETN--MLSELETESGKTFFGVFAL 225            38
troutTNF2    QLNEGDKLWTETN--RLTDVEPEQGKNFFGVFAL 255            36
troutTNF1    QLNEGDKLWTETN--RLTDVEPEQGKNFFGVFAL 246            36
mouseTNF     QLEKGDQLSAEVNLPKYLDFAE-SGQVYFGVIAL 235            28
             :*.  *:* :         .    *: :**.::*
```

Figure 4.4 Amino acid alignment of goldfish TNFα-2 with sequences from carp, catfish, seabream, flounder trout and mouse. Fully conserved residues are indicated by an asterisk (*), partially conserved and semi-conserved substitutions are represented by ":" and "." respectively. The percent identities are reported following the alignment. The trans-membrane domain (**bold** and underlined), cleavage site (↘), TNF signature sequence (highlighted in grey), and conserved cysteines (✧) are all indicated on the goldfish sequence.

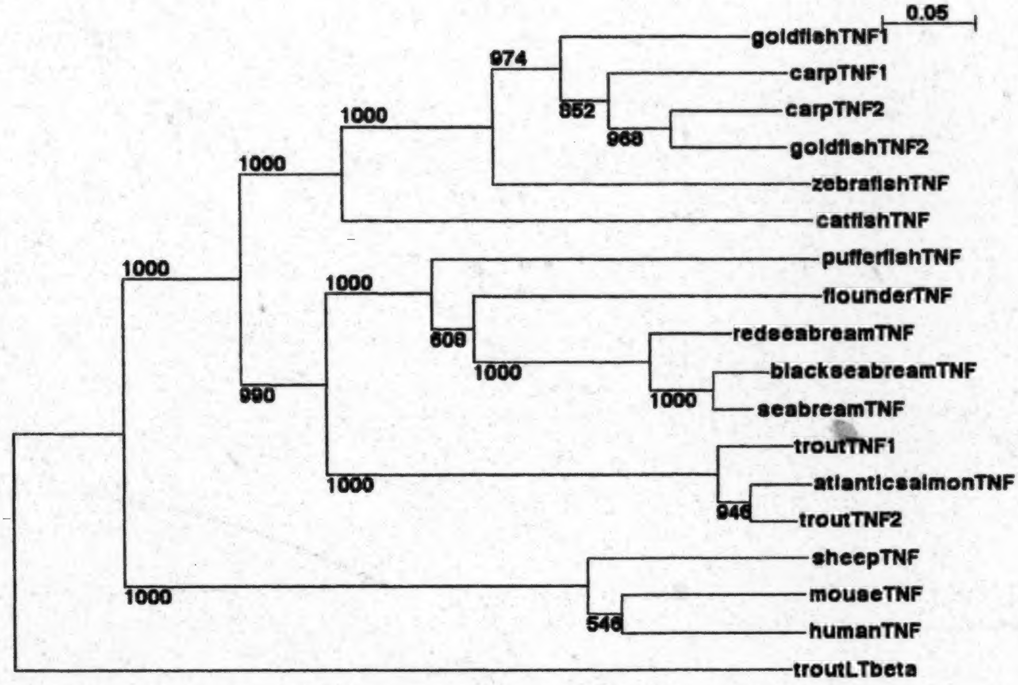
0.05
goldfishTNF1
carpTNF1
carpTNF2
goldfishTNF2
zebrafishTNF
catfishTNF
pufferfishTNF
flounderTNF
redseabreamTNF
blackseabreamTNF
seabreamTNF
troutTNF1
atlanticsalmonTNF
troutTNF2
sheepTNF
mouseTNF
humanTNF
troutLTbeta
974
852
968
1000
1000
1000
1000
608
1000
1000
990
1000
946
1000
546

Figure 4.5 Phylogenetic analysis of goldfish TNFα-1 and 2 isoforms as compared to TNFα sequences from other fish and mammals. Trout lymphotoxin beta (troutLTbeta) was used as an out-group. The analysis was conducted using the neighbor joining method (Clustal X) and was bootstrapped 1000 times. The bootstrap values are reported for each branch point.

atgatgatagatgttgagagtcagttagctgaagaaggagcgcaggtgacggtgtcgaggaggaggtccggt
gtctggcgggtgtgtggggtcctgctggctgtggccctgtgtgccgccgctgctgtctgcttcacattcaacaagt
ctcagaacaatcaggaaagtggaaatgagctgaggctcaca**//**ttaagagaccatctttcaaaagaaaatgtca
ct**tccaaggccgcgatccattta**acaggtgcatatgaccctgacgtgtgtacggacaacctagactggaaaca
gaaccaggaccaggcttttgtttcaggtggcttgaaactagtggacagagagatcatcattccttacgacggcatt
tacttcgtctacagtcaggtgtctttccacatcagctgcaaggctgacgtgactgaggaacacgagggcgtgca
catgagccacgcagtgttgcgcttctcggagtcctacgccagctacaagccgctcttcagcgcgatccgctcgg
cctgcgtgcacgcgactgacactgaagacctgtggtacaacaccatttacctcggagcggccttcaacctgcga
gctggagacaaactgcgcaccgacaccaccacagaactcctgccccgcgtggaaagcgaaaacggaaaga
cc**ttcttcggggtgttt**gctttatga

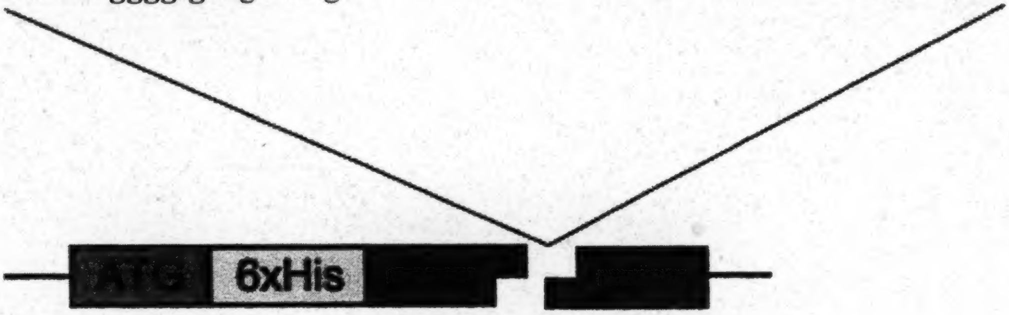

Figure 4.6 Schematic showing the recombinant TNFα-2 sequences (black) that was cloned into the pET SUMO vector containing an N-terminal 6xHis tag and the SUMO secretion signal. The rest of the TNFα-2 sequence is shown in grey. The predicted cleavage site was also denoted by (//).

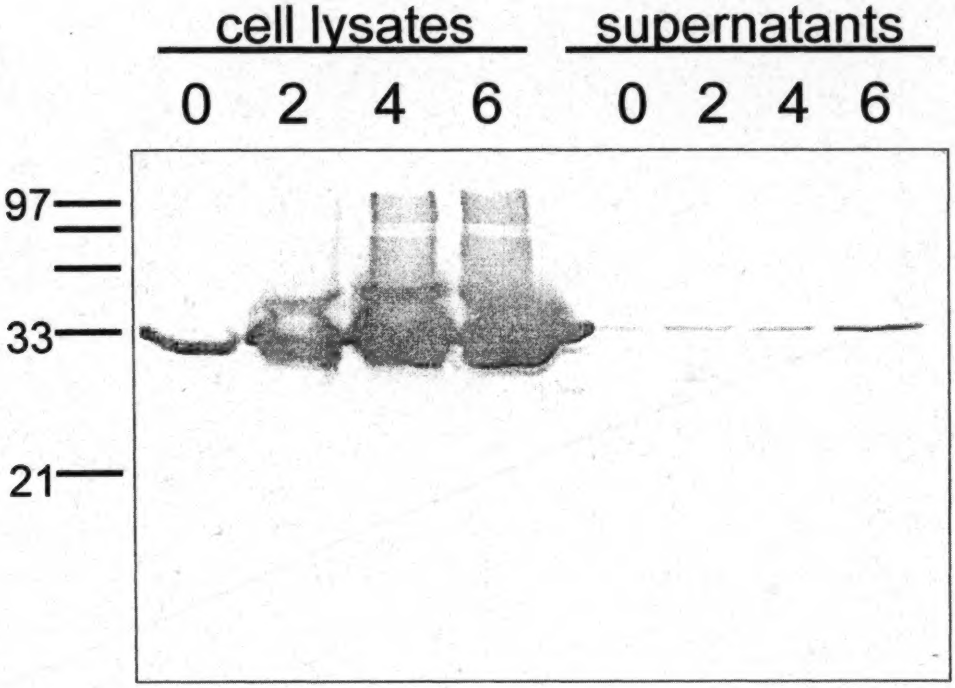
cell lysates
supernatants
0
2
4
6
0
2
4
6
97
33
21

Figure 4.7 Western blot showing the expression of recombinant goldfish TNFα-2 in the cell lysates and supernatants after induction with IPTG for 0, 2, 4, and 6 hr. Recombinant TNFα-2 was detected using an anti-HIS antibody.

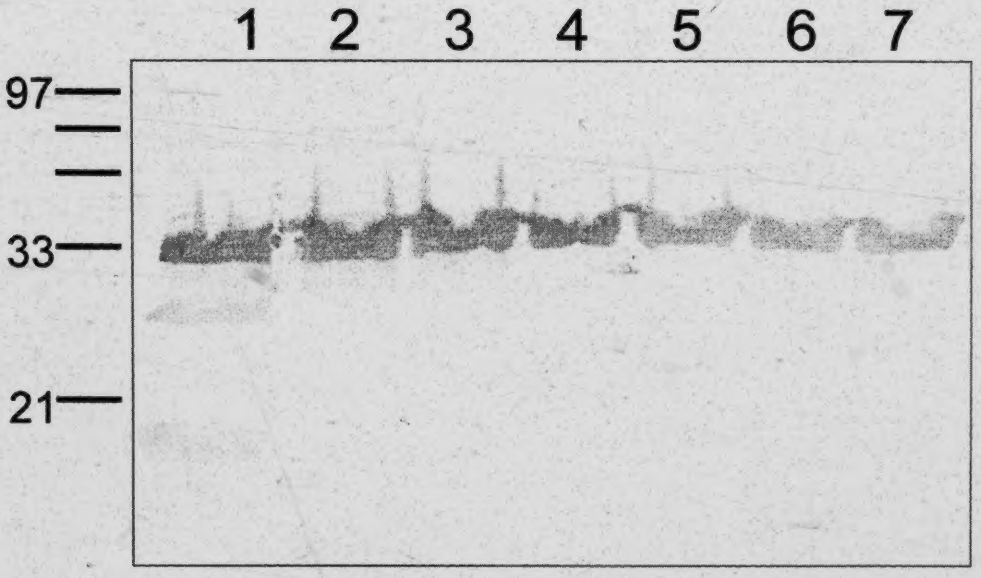

Figure 4.8 Western blot showing the detection of purified goldfish TNFα-2 in 7 elution fractions. Recombinant TNFα-2 was detected using an anti-HIS antibody.

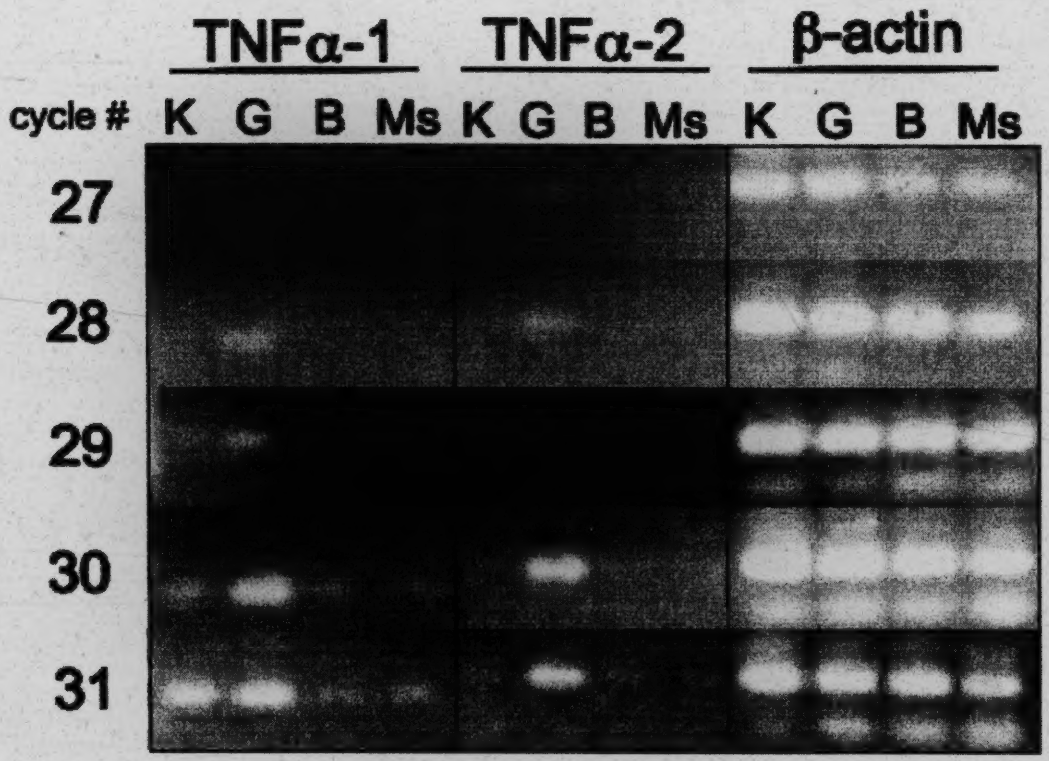
TNFα-1
TNFα-2
β-actin
cycle #
K G B Ms K G B Ms K G B Ms
27
28
29
30
31

Figure 4.9 RT-PCR showing the mRNA expression of goldfish TNFα-1, TNFα-2 and β-actin in the kidney (K), gill (G), brain (B) and muscle (Ms) of goldfish. The levels of detection are shown after 28 and 31 PCR amplification cycles. A representative gel is shown.

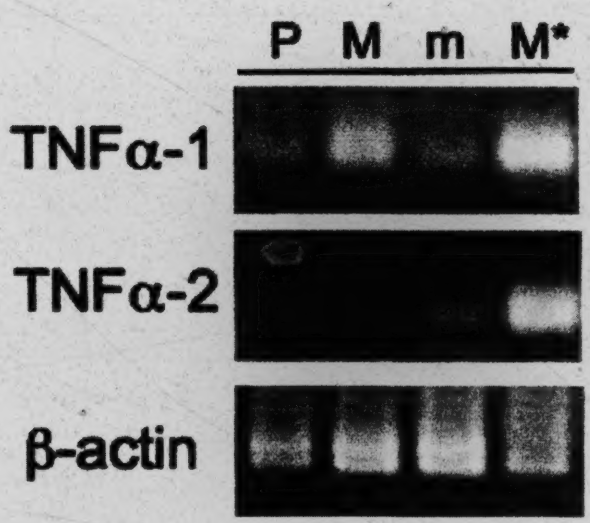
P
M
m
M*
TNFα-1
TNFα-2
β-actin

Figure 4.10 RT-PCR showing the mRNA expression of goldfish β-actin TNFα-1, and TNFα-2 in sorted goldfish progenitor (P), mature macrophages (M), monocytes (m) and activated macrophage cultures (M^*). cDNA 6 pooled from six fish.

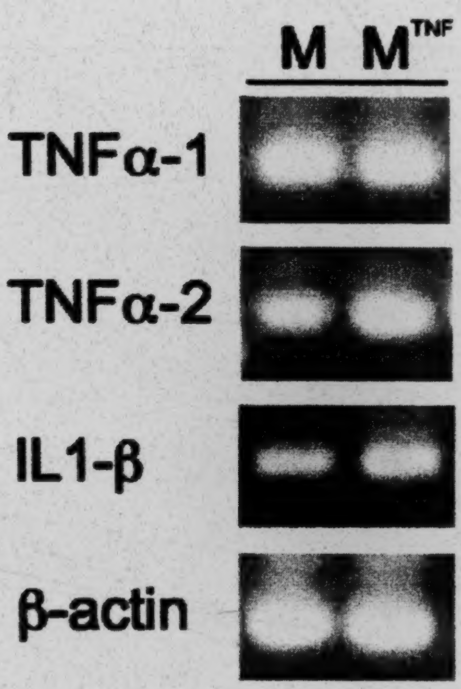
M
M^TNF
TNFα-1
TNFα-2
IL1-β
β-actin

Figure 4.11 RT-PCR showing the mRNA expression of goldfish TNFα-1, and TNFα-2, IL1-β and β-actin in un-stimulated (M) or recombinant TNFα-2 stimulated mature macrophages (M^{TNF}). cDNA from pooled RNA from 3 fish.

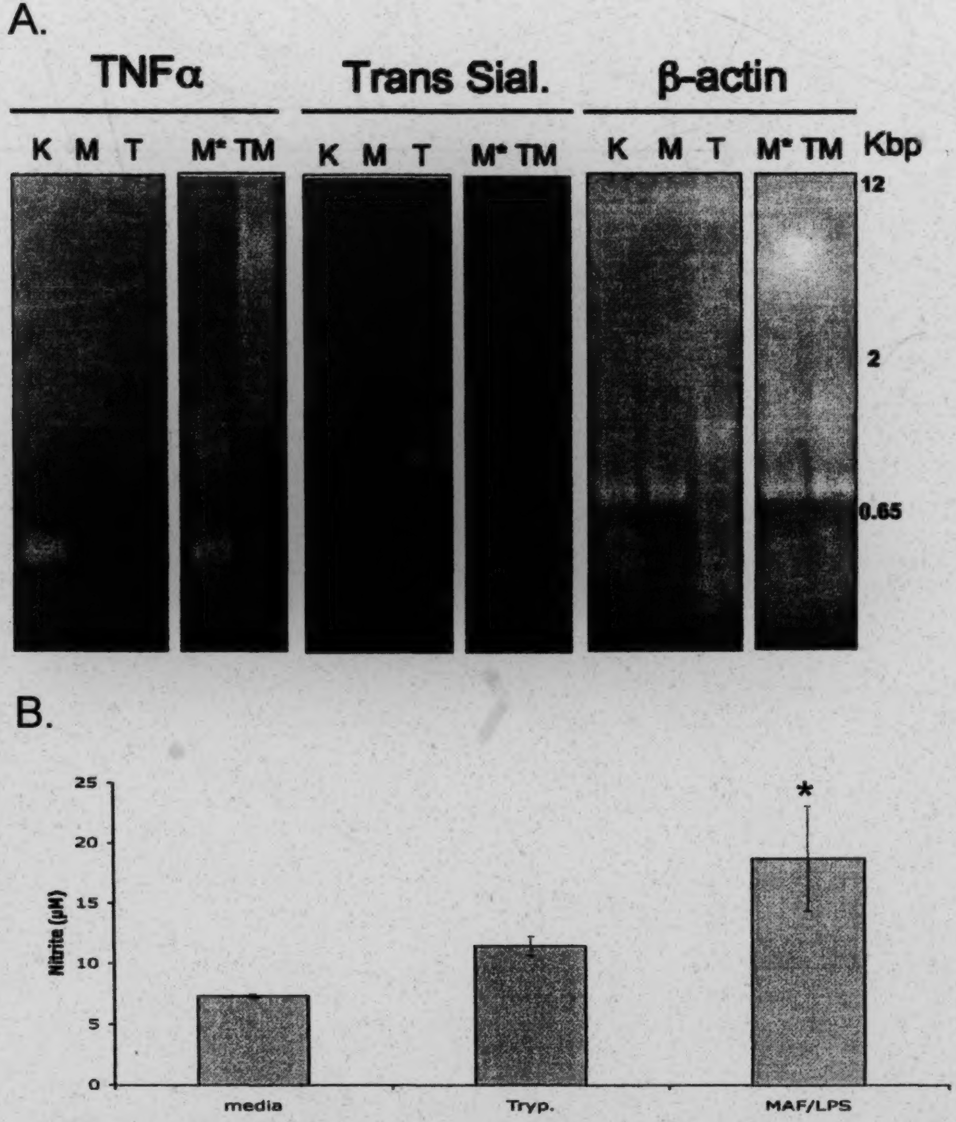

Figure 4.12 A. RT-PCR showing the expression of β-actin, TNFα-2 and Trans sialidase (an internal control for the presence of parasites) in goldfish kidney (K), un-stimulated macrophage culture (M), trypanosome cultures (T), MAF & LPS stimulated cultures (M^*) and trypanosome/macrophage co-incubation cultures (TM). Representative of 3 fish. B. Nitric oxide response of goldfish macrophages to medium alone, trypanosome (Tryp.) stimulation and mitogen stimulated macrophage supernatants and bacterial endotoxin (MAF & LPS) stimulation. Only MAF & LPS induced a significant response compared to the negative control as indicatied by asterick (*).

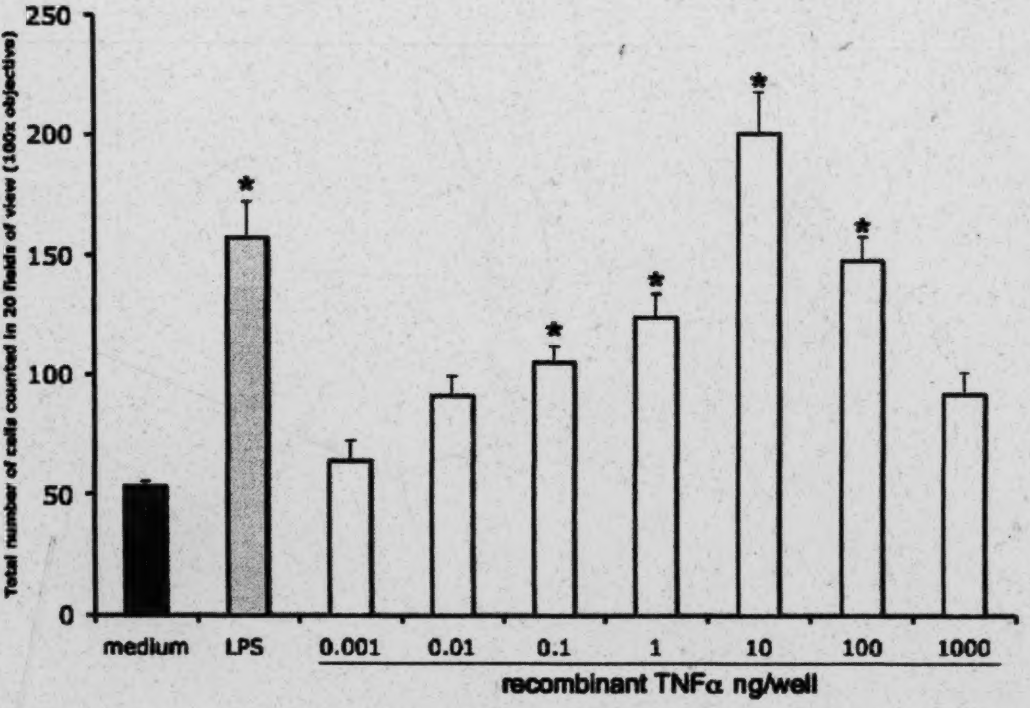

Figure 4.13 Chemotactic response of goldfish macrophages to culture medium, 5 ng/well bacterial endotoxin (LPS) and recombinant goldfish TNFα-2 (0.001, 0.01, 0.1, 1, 10, 100 and 1000 ng/well). Treatments with responses that are statistically different from the medium control are indicated by an asterisk (*). Cells were isolated from 6 individual fish. Experiment repeated twice.

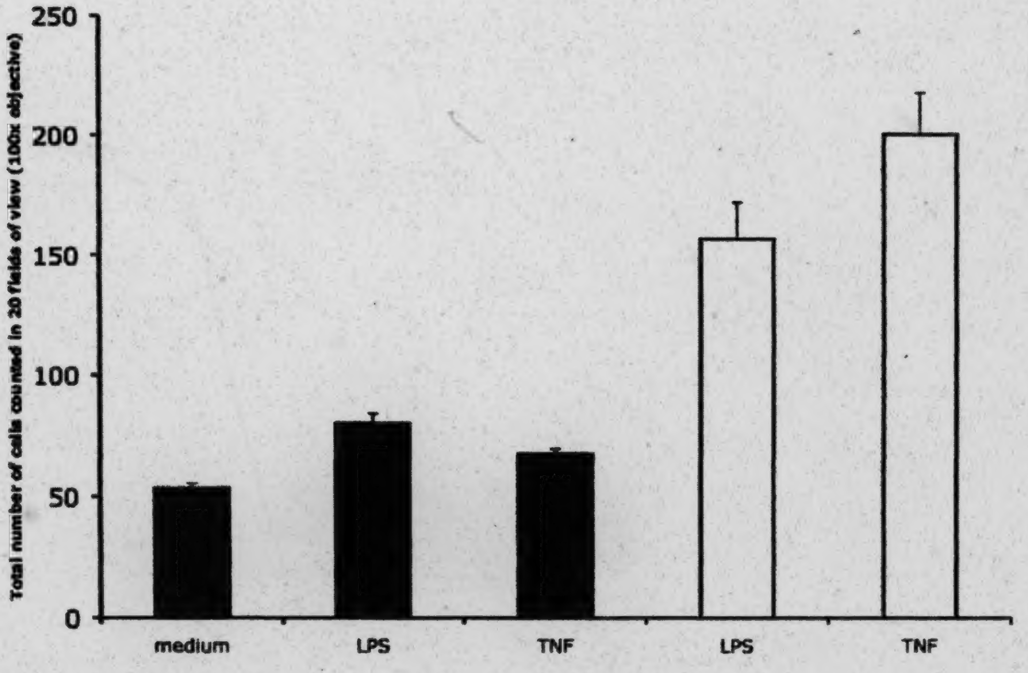

Figure 4.14 The chemokinetic response of goldfish macrophages to medium alone, bacterial endotoxin (5 ng/well) or recombinant goldfish TNFα-2 (TNF 10 ng/well) in both the upper and lower wells (gray bars). White bars: chemokinetic LPS (5 ng/well) or TNFα-2 (10 ng/well) in the lower well alone (clear bars).

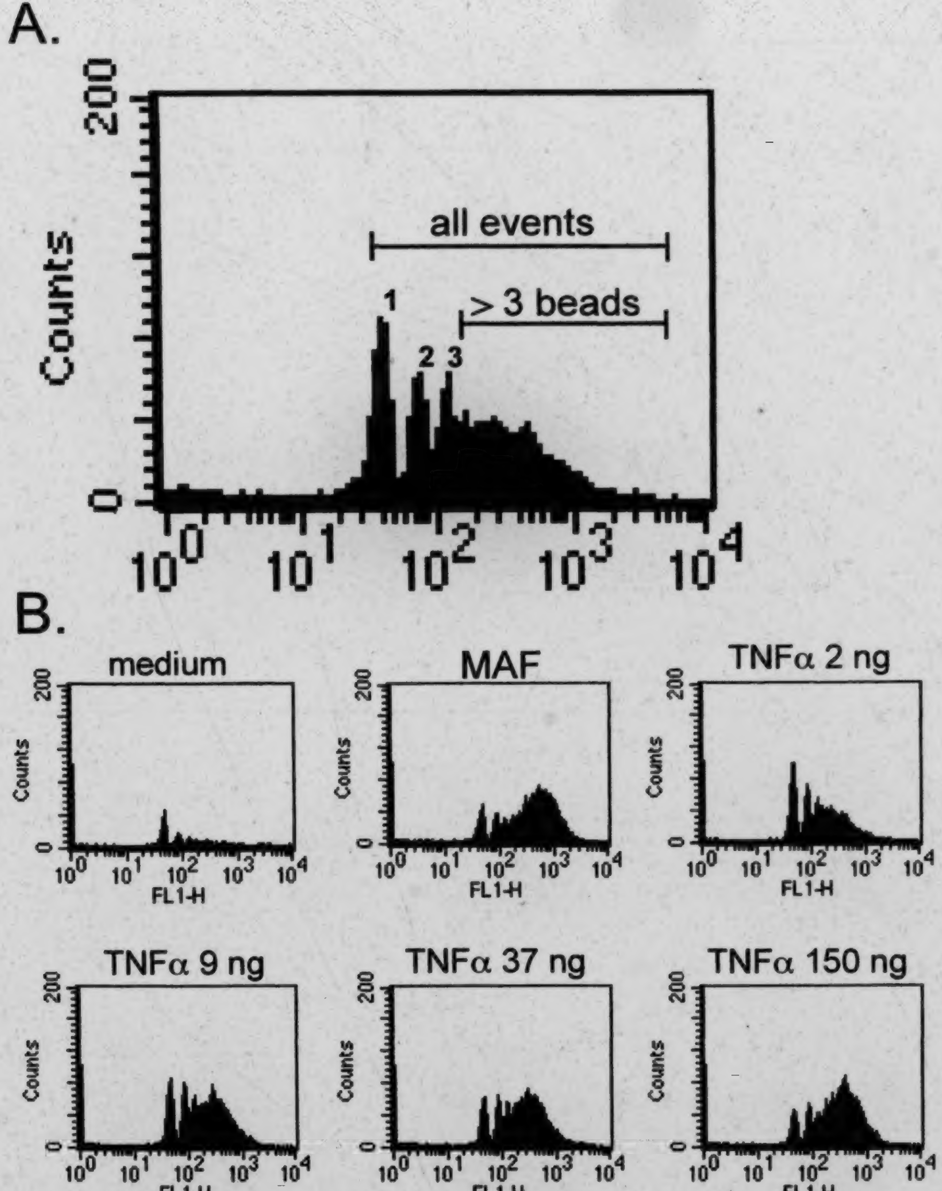

Figure 4.15 A. A fluorescence histogram depicting those peaks representing cells having ingested 1, 2, or 3 beads as well as the gates representing all cells with ingested beads and those with >3 beads. B. Representative fluorescence histograms from one fish stimulated with culture medium, mitogen stimulated macrophage supernatants (MAF), or recombinant goldfish TNFα-2 (2, 9, 37, and 150 ng/well).

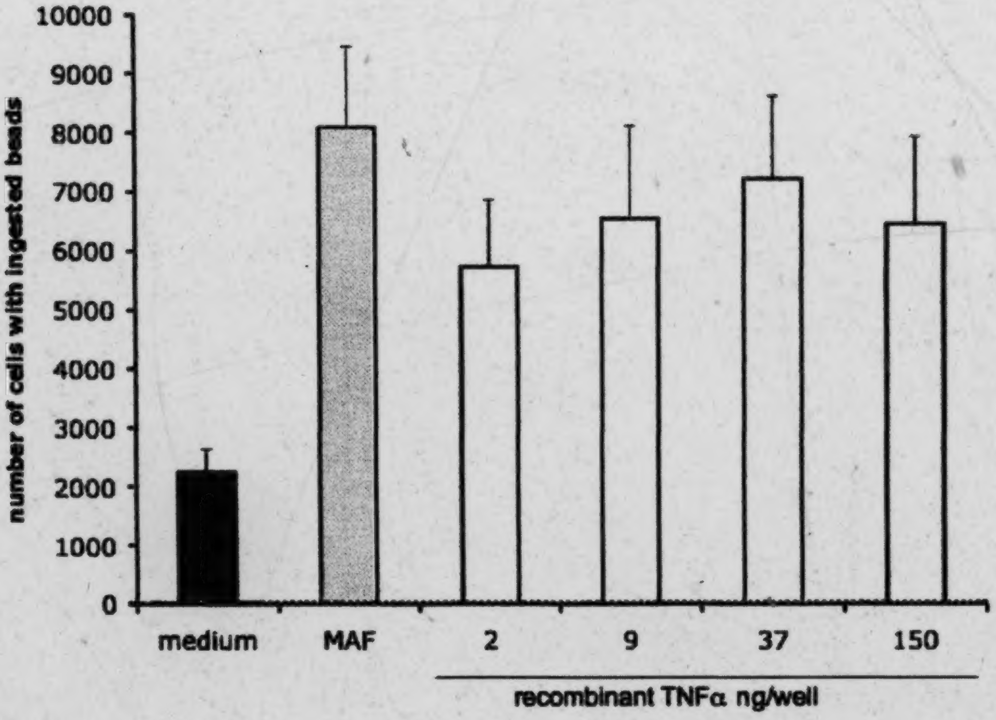

Figure 4.16 The phagocytosis of beads by goldfish macrophages stimulated with medium alone, mitogen stimulated macrophage supernatants (MAF) or recombinant goldfish TNFα-2 (2, 9, 37, and 150 ng/well). For each of 6 fish 30, 000 events/cells were analyzed. Experiments were done using mixed macrophage cultures that also contained progenitor and monocytes subpopulations. Experiment repeated twice.

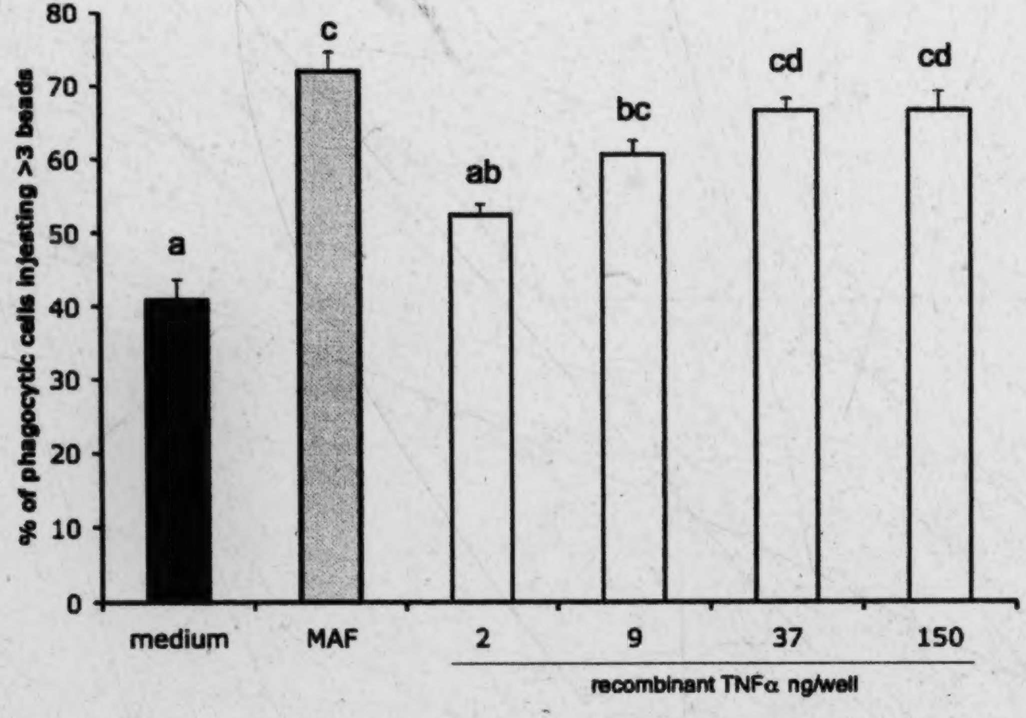

Figure 4.17 The percent of phagocytic cells having ingested >3 beads after stimulation with culture medium, mitogen stimulated macrophage supernatants (MAF) and recombinant goldfish TNFα-2 (2,9,37, and 150 ng/well). The different letters "a, b, c, or d" denote groups determined to be significantly different by Tukey's test. Cells were isolated from 6 individual fish. Cells were isolated from 6 fish. Experiment was repeated twice.

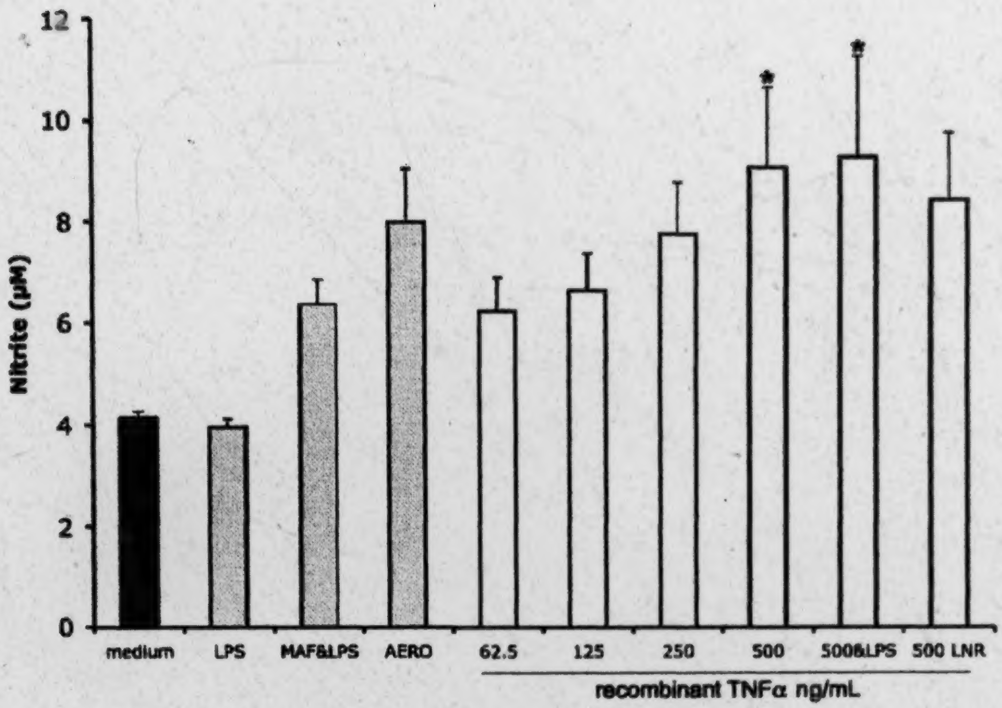

Figure 4.18 Nitric oxide response of goldfish macrophages to culture medium, bacterial endotoxin (LPS 1 µg/mL), mitogen stimulated macrophage supernatants and LPS (MAF&LPS), *Aeromonas* (AERO), and recombinant goldfish TNFα-2 (62.5, 125, 250, 500 ng/mL). Also included are 500 ng/mL TNFα-2 & LPS treatment (500&LPS) and a 500 ng/mL treatment of goldfish TNFα-2 prior to LPS removal (500 LNR). Asterisks (*) indicate those treatments that were significantly different from medium alone or LPS as determined using Tukey's test. Cells were isolated from 5 individual fish. Experiment repeated twice.

CHAPTER 5[1]

CLONING AND EXPRESSION ANALYSIS OF PROMININ OF THE GOLDFISH

5.1 Introduction

In humans, cells of the mononuclear phagocytic lineage originate in the bone marrow from a common myeloid progenitor that is shared with neutrophils [139]. In fish the major hematopoietic organ is the kidney. Macrophage progenitor cells can be isolated from goldfish kidneys and have been shown to differentiate into mononuclear cells [140]. Monocytes that arise from the hematopoietic tissues circulate in the peripheral blood before being recruited into the tissues to become macrophages. This process occurs as both a part of homeostasis as well as part of an inflammatory response [139]. Therefore, it is important for immunologists to have an understanding of macrophage development beginning with the identification of their precursors.

The integral membrane protein known as prominin-1 was first identified on the apical surface of mouse neural epithelial cells that function as precursors to neurons and macroglial cells [141]. Subsequent investigation revealed that prominin-1 and a protein found on the surface of hematopoietic progenitor cells recognized by the antibody designated as AC133, were homologous proteins [3, 142]. Prominin-like proteins have been identified in mammals [3, 141] and across the metazoan kingdom including *C.elegans* [141], *Drosophila* and chicken and zebrafish [143],

Prominin-1 specifically localizes to envaginated regions of the cell membrane, including the microvilli of epithelial cells as well as the simple membrane protrusions of hematopoietic progenitor cells [142]. Both mouse and human prominin-1 are integral membrane proteins with five transmembrane domains with alternating small cytoplasmic and large extracellular loops. The large extracellular loops (>200 residues each) also possess several N-glycosylation sites which may be of particular importance to the immunoreactivity of the AC133 antibody with certain cell types but not others [142, 144].

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[1] A version of this chapter has been published: Walsh JG, Barreda D, and M Belosevic. Cloning and Expression Analysis of Goldfish (*Carassius auratus* L.) Prominin. Fish and Shellfish Immunology. In press (April 2006)

Another prominin molecule, prominin-2, has been recently identified and is an ortholog of prominin-1 [143]. Although the sequence identity between prominin-1 and 2 was low (~30%), the genome arrangement of the two prominins was similar, suggesting a common ancestral gene for the two forms. Structurally, prominin-2 shows the same membrane topology with five transmembrane domains and two large N-glycosylated extracellular loops[143].

Expression analysis of prominin-1 has revealed a close association with epithelial cell types as well as several progenitor/stem cell populations. The presence of alternatively spliced isoforms has complicated the tissue expression analyses, however some isoforms appear to be quite ubiquitously expressed [145-146].

Prominin-1 has been used as a marker of different subpopulations of stem cells, hematopoietic progenitor cells, endothelial progenitor cells (CD34-positive cells), epithelial cells and myogenic cells [141, 147-150]. The prominin-1 isoform (AC133-2), thought to specifically mark hematopoietic stem cells, has recently been found to have a broad pattern of tissue expression, suggesting that the ability of the AC133 antibody to identify stem cell progenitors may not be limited to hematopoietic tissues [145].

Little is known about the biological function of prominin-1. A recessive disorder resulting in retinal degeneration was linked to a mutation in the prominin-1 gene. The mutation resulted in a truncated protein that failed to translocate to the cell surface. Absence of prominin-1 from the membrane envaginations that precede the formation of photoreceptive disk membranes in rod cells was thought to cause retinal degeneration. These findings have led to the hypothesis that prominin-1 function was the maintenance of structures based on envaginations of the membrane [147]. Interestingly, prominin-2 was not expressed in the retina and defects caused in the eye by the absence of prominin-1 do not appear to occur in other tissues where prominin-1 and prominin-2 expression overlapped [143].

In this chapter, I describe the cloning, characterization and tissue expression analysis of a prominin-like gene of the goldfish.

5.2 Results

5.2.1 Sequence analysis

The open reading frame of goldfish prominin encodes an 837 amino acid protein with a predicted molecular weight of ~93 kD. Hydropathy analysis predicts six hydrophobic regions the most N-terminal of which is likely a signal peptide and is associated with a predicted cleavage site located after glycine 27 (Figure 5.1 arrow). The remaining predicted hydrophobic regions indicate that goldfish prominin like its mammalian counterparts has five transmembrane domains, an extracellular N-terminus and a cytoplasmic C-terminus. In keeping with this shared topology is the presence of two large extracellular loops of 218 and 271 amino acids respectively (Figure 5.1). The sequence has been submitted to GenBank (accession No. **DQ233501**)

Software analysis predicted 10 potential N-glycosylation sites located on the extracellular loops of goldfish prominin supporting the hypothesis that it like its mammalian counterparts is a glycosylated protein. Goldfish prominin also possesses the same cysteine rich region located just after the first transmembrane domain that has been observed in other prominin sequences (Figure 5.1). Also identified in the goldfish sequence is the prominin signature as proposed by Fargeas *et al*. [143], CXPX(12/13)CX(5)(P/S)X(4)WX(2)hX(4)hhXh, where X stands for any residue, h stands for any hydrophobic residue and residues in parentheses indicate possible alternatives at that position. The number of residues is also indicated in parentheses.

The top three matches of a BLASTP search of goldfish prominin are all predicted prominin-like proteins from zebrafish (XP 684527, XP 688810, and AAK54609). In order to gain information on the relatedness of goldfish prominin to other vertebrate prominins simple percent identities were calculated (Table 5.1). Within the mammals prominin sequences can be grouped as type 1 or type 2 based on sequence identity. Type 1 prominins share as much as 59% identity between species and similarly there is moderately high conservation within the type 2s. However, the shared identity between the two types is relatively low (<30%). Certain zebrafish sequences appear to be more closely related to the mammalian type 1s (~40%) than type 2s (<30%) indicating the type 1 and 2 subfamilies may also be present in fish. However, goldfish prominin and other fish prominins appear not to share particularly similarity with either of the mammalian subtypes the identity scores for both between 27-29% (Table 5.1). This analysis also suggests that goldfish prominin is closely related to an unidentified protein from the spotted pufferfish *Tetraodon nigroviridis* (CAF91648) (Table 5.1). Based on blast results as well as the presence of the prominin signature sequence (data not shown) it seems likely that this unnamed protein represents a prominin homolog from pufferfish.

5.2.2 *Southern blot analysis*

A southern blot of the goldfish genomic DNA was conducted to detect the presence of homologous isoforms of goldfish prominin. Kpn I which was predicted not to have any cut sites within the probe spanning region suggested the presence of a single copy gene. DNA treated with Sph I that was predicted to have one cut site within the probed region revealed the predicted 2 bands for a single copy gene. DNA treated with Xba I which was not predicted to cut within the probe revealed 3 bands. The combined size of the lower two bands approximated the size of the upper band indicating that a single cut site is differentially present on two alleles. The combined evidence suggests that goldfish prominin is a single copy gene (Figure 5.2).

5.2.3 Prominin mRNA expression in goldfish tissues and sorted goldfish macrophages

In order to determine the expression levels of goldfish prominin in whole tissues, a real time PCR analysis of 7 tissues was conducted including the kidney, gill liver, spleen, heart, brain and intestine. All tissues examined expressed goldfish prominin however both gill and kidney showed evidence of an elevated level of expression (Figure 5.3).

To determine the pattern of expression in a population of hematopoietic cells real time PCR analysis was preformed using total RNA from sorted goldfish macrophages. This analysis compared the expression levels between three subpopulations of sorted goldfish macrophages: early progenitors, monocytes and mature macrophages. Goldfish prominin was expressed at some level in all subpopulations of *in vitro*-derived macrophages . However, the highest levels of expression were observed in the progenitors followed by the mature macrophages. Monocytes appeared to express prominin only at low levels (Figure 5.4 B). A similar trend was observed through conventional RT-PCR (Figure 5.4 A).

5.3 Discussion

Prominin-like proteins are highly conserved, and in addition to mammals [3, 141] have been found in an array of organisms including *C.elegans* [141], *Drosophila* [143], chicken [143], zebrafish [143] and now in the goldfish. The relationship of goldfish prominin to these other prominin-like proteins was supported by sequence analysis. The mammalian prominins divide into what appear to be two orthologous groups (prominin-1 type and prominin-2 type). Although certain zebrafish sequences share greater identity with the prominin-1 type, goldfish prominin does not appear to fall to any particular side of this dichotomy This is exemplified by the relatively low identity score when compared to both type 1 and type 2 mammalian prominins.

Although prominin has gained widespread use as a marker of stem/progenitor cell populations, its biological roles are not fully understood. A retinal degeneration disorder linked to a mutation in the prominin-1 gene has led some researchers to speculate a role for prominin in the formation and maintenance of membrane envaginations perhaps through the maintenance of novel cholesterol based membrane microdomains [146, 151-152]. It as also been proposed that the localization of prominin to the plasma membrane suggests a role in cell-cell interactions or ligand-receptor interactions [144, 152-153]. At present, the possible functions of the prominins in teleosts can only be deduced based on the findings reported for mammalian systems. The high degree of conservation of prominins amongst the metazoans, suggests that there may be some fundamental requirement underpinning a conservation of their function.

It has been well established that in mammals both prominin-1 and 2 exist in multiple isoforms as a result of alternative splicing. This may play a role in the diversity of fish prominins as well. Southern blot analysis of goldfish prominin indicates that it is a single coply gene and that there does not appear to be any highly homologous isoforms encoded on separate genes. However, based on the low degree of homology between the mammalian type-1 and type-2 prominins as well as the number of reported sequences for zebrafish we don't expect that this is the only prominin like gene of goldfish.

Similar to its mammalian homologs, goldfish prominin appears to be arranged as a pentaspan membrane protein. The goldfish protein possesses several N-glycosylation sites located on two large extracellular loops. Variation in the glycosylation pattern on mammalian prominins has been of critical importance in defining the AC133 epitope that marks certain stem cell populations. Conserved cysteine residues were also found in the goldfish prominin sequence, the most notable being the conservation of a cysteine rich region located just terminal to the first transmembrane domain. The prominin signature as proposed by Fargeas et al. [143] was also present in the goldfish sequence.

Real time PCR analysis indicated that prominin was expressed in all seven goldfish tissues examined, which was similar to that of the AC133-2 isoform expression of mammals [145]. The expression of prominin in the gill and kidney was higher than in other tissues. Although prominin has been described as being characteristically located to plasma membrane protrusions, its tissue expression was more variable and has been shown to differ significantly between different isoforms of the same organism [145, 154]. The precise nature of prominin expression in the kidney and gills cannot be known at this time. As the major hematopoietic organ in fish expression in the kidney is very likely to be at least in part linked to resident macrophage cells and their progenitors. It is also possible that the level of expression seen in the gills is linked to a sequestering of immune type cells within these tissues however the higher level of expression could also be due to the relatively high rate of cellular turnover in the gill.

In light of the importance of human prominin as a marker of stem cells we also examined the expression of prominin in three *in vitro*-derived macrophage subpopulations. These stages included early progenitors, monocytes and mature macrophages. Our analyses indicate that goldfish prominin was most highly expressed in the progenitor subpopulation, suggesting that prominin may mark the hematopoietic progenitors of teleosts similar to mammals. It is interesting to note that the expression of goldfish prominin was only slightly lower in the mature macrophage subpopulation. Whether prominin has a functional role in teleost macrophage development remains to be determined. It is possible that the progenitor and mature macrophage populations being normally located to the tissues may have a differing functional requirement for prominin expression than the circulating monocytes. It is also possible that prominin may be a marker for an alternate macrophage developmental pathway (from progenitor directly to macrophage) which we have shown previously to be important for the generation and self-renewal of goldfish tissue macrophages [140]. Finally it has been reported in mammalian systems that there may be local proliferation of mature macrophages within tissues [139] suggesting that mature macrophages may possess properties previously though to be exclusive to their progenitor source population.

Higher expression of prominin in the progenitor cell populations suggests that an AC133-like epitope may be present in fish. In addition to its utility as a marker of early progenitor cells, prominin may also have an active role in the regulation of other aspects of fish development.

```
    atg ctc aac atg agg tcc aga atg aaa aag gtt
  1 --- --- --- +-- --- --- -+- --- --- --+ ---
    tac gag ttg tac tcc agg tct tac ttt ttc caa
    Met Leu Asn Met Arg Ser Arg Met Lys Lys Val

    ttt tgg agt ggc ttt ttg ctc ctg ttt ctc tgg
 34 --- --- +-- --- --- -+- --- --- --+ --- ---
    aaa acc tca ccg aaa aac gag gac aaa gag acc
    Phe Trp Ser Gly Phe Leu Leu Leu Phe Leu Trp

    aca ttg gta aat gga caa gtg tcc tgt cct gta
 67 --- +-- --- --- -+- --- --- --+ --- --- ---
    tgt aac cat tta cct gtt cac agg aca gga cat
    Thr Leu Val Asn Gly↘Gln Val Ser Cys Pro Val

    gag acg acg aga aca gcg ctc caa act cca gat
100 +-- --- --- -+- --- --- --+ --- --- --- +--
    ctc tgc tgc tct tgt cgc gag gtt tga ggt cta
    Glu Thr Thr Arg Thr Ala Leu Gln Thr Pro Asp

    gaa ata cca acc aag ctg acc ttc aac agt gcc
133 --- --- -+- --- --- --+ --- --- --- +-- ---
    ctt tat ggt tgg ttc gac tgg aag ttg tca cgg
    Glu Ile Pro Thr Lys Leu Thr Phe Asn Ser Ala

    ttt atg tct cca atg gta cat tca ttc ctg ggc
166 --- -+- --- --- --+ --- --- --- +-- --- ---
    aaa tac aga ggt tac cat gta agt aag gac ccg
    Phe Met Ser Pro Met Val His Ser Phe Leu Gly

    tct gtc cag tcc aat cca ttc cct aaa gat ata
199 -+- --- --- --+ --- --- --- +-- --- --- -+-
    aga cag gtc agg tta ggt aag gga ttt cta tat
    Ser Val Gln Ser Asn Pro Phe Pro Lys Asp Ile

    ctc att gga atc aca aaa ggt tac gac aca gag
232 --- --- --+ --- --- --- +-- --- --- -+- ---
    gag taa cct tag tgt ttt cca atg ctg tgt ctc
    Leu Ile Gly Ile Thr Lys Gly Tyr Asp Thr Glu

    acg gtt aaa gag gtt ttg caa tac cag aca gga
265 --- --+ --- --- --- +-- --- --- -+- --- ---
    tgc caa ttt ctc caa aac gtt atg gtc tgt cct
    Thr Val Lys Glu Val Leu Gln Tyr Gln Thr Gly
    ttc ttg gtc tgc gtg gcc att ggg atc ctg tac
298 --+ --- --- --- +-- --- --- -+- --- --- --+
    aag aac cag acg cac cgg taa ccc tag gac atg
    Phe Leu Val Cys Val Ala Ile Gly Ile Leu Tyr

    att gtc ctg atg cct cta att ggc ctg ttc ttt
331 --- --- --- +-- --- --- -+- --- --- --+ ---
    taa cag gac tac gga gat taa ccg gac aag aaa
    Ile Val Leu Met Pro Leu Ile Gly Leu Phe Phe

    gcc tgc tgt cgt tgc tgt ggt aac tgc ggg ggc
364 --- --- +-- --- --- -+- --- --- --+ --- ---
    cgg acg aca gca acg aca cca ttg acg ccc ccg
    Ala Cys Cys Arg Cys Cys Gly Asn Cys Gly Gly
         *   *       *   *           *

    cgc atg cag cag aag aaa aca aca aac atc cac
397 --- +-- --- --- -+- --- --- --+ --- --- ---
    gcg tac gtc gtc ttc ttt tgt tgt ttg tag gtg
    Arg Met Gln Gln Lys Lys Thr Thr Asn Ile His

    tgc aag cga aga agc ttt tac ttg tgc aca ttt
430 +-- --- --- -+- --- --- --+ --- --- --- +--
    acg ttc gct tct tcg aaa atg aac acg tgt aaa
    Cys Lys Arg Arg Ser Phe Tyr Leu Cys Thr Phe

    ctc atc act gta cta att atg gca ggg aac atc
463 --- --- -+- --- --- --+ --- --- --- +-- ---
    gag tag tga cat gat taa tac cgt ccc ttg tag
    Leu Ile Thr Val Leu Ile Met Ala Gly Asn Ile

    tgc gtg ttc ctg agc agt aca aac act tca gag
496 --- -+- --- --- --+ --- --- --- +-- --- ---
    acg cac aag gac tcg tca tgt ttg tga agt ctc
    Cys Val Phe Leu Ser Ser Thr Asn Thr Ser Glu
                                 ^
    act ttg atg cgc agc ccc aca gaa ctc acc gac
529 -+- --- --- --+ --- --- --- +-- --- --- -+-
    tga aac tac gcg tcg ggg tgt ctt gag tgg ctg
    Thr Leu Met Arg Ser Pro Thr Glu Leu Thr Asp

    atc ttg gag aat gtc aaa ggc tat ctt aac tcc
529 --- --- --+ --- --- --- +-- --- --- -+- ---
    tag aac ctc tta cag ttt ccg ata gaa ttg agg
    Ile Leu Glu Asn Val Lys Gly Tyr Leu Asn Ser
    att cct cag caa atc caa caa gtg acg aat gag
562 --- --+ --- --- --- +-- --- --- -+- --- ---
    taa gga gtc gtt tag gtt gtt cac tgc tta ctc
    Ile Pro Gln Gln Ile Gln Gln Val Thr Asn Glu
                                         ^

    agc cgt gtg act gtg gat acg gtc aaa aac aac
595 --+ --- --- --- +-- --- --- -+- --- --- --+
    tcg gca cac tga cac cta tgc cag ttt ttg ttg
    Ser Arg Val Thr Val Asp Thr Val Lys Asn Asn

    tta gtt gaa aca ggg cct ttg ctt ggc aaa ctg
628 --- --- --- +-- --- --- -+- --- --- --+ ---
    aat caa ctt tgt ccc gga aac gaa ccg ttt gac
    Leu Val Glu Thr Gly Pro Leu Leu Gly Lys Leu

    atc cag aat ggt ctt aag ggc cac ctt gac cca
661 --- --- +-- --- --- -+- --- --- --+ --- ---
    tag gtc tta cca gaa ttc ccg gtg gaa ctg ggt
    Ile Gln Asn Gly Leu Lys Gly His Leu Asp Pro

    gcc tta aac tcc act act gaa ata ggc caa gtg
694 --- +-- --- --- -+- --- --- --+ --- --- ---
    cgg aat ttg agg tga tga ctt tat ccg gtt cac
    Ala Leu Asn Ser Thr Thr Glu Ile Gly Gln Val
             ^

    ata aac agc acc agc gat gga ctg ctg cag ctt
727 +-- --- --- -+- --- --- --+ --- --- --- +--
    tat ttg tcg tgg tcg cta cct gac gac gtc gaa
    Ile Asn Ser Thr Ser Asp Gly Leu Leu Gln Leu
         ^

    aat aaa acc ttg gga gct gct gaa acc gaa ggc
760 --- --- -+- --- --- --+ --- --- --- +-- ---
    tta ttt tgg aac cct cga cga ctt tgg ctt ccg
    Asn Lys Thr Leu Gly Ala Ala Glu Thr Glu Gly
     ^

    ggc cgc gct gca agc tat tta tct gcg gtg agg
793 --- -+- --- --- --+ --- --- --- +-- --- ---
    ccg gcg cga cgt tcg ata aat aga cgc cac tcc
    Gly Arg Ala Ala Ser Tyr Leu Ser Ala Val Arg

    cag agg att aac aac act ttg cgt atg tca gac
826 -+- --- --- --+ --- --- --- +-- --- --- -+-
    gtc tcc taa ttg ttg tga aac gca tac agt ctg
    Gln Arg Ile Asn Asn Thr Leu Arg Met Ser Asp
                 ^
     tgt gta aac tgc gct gcc ctt cag cca gaa ctg
 859 --- --- --+ --- --- --- +-- --- --- -+- ---
     aca cat ttg acg cga cgg gaa gtc ggt ctt gac
     Cys Val Asn Cys Ala Ala Leu Gln Pro Glu Leu

     gat aaa cta tca ctg gat gga agc ctt gaa ttt
 892 --- --+ --- --- --- +-- --- --- -+- --- ---
     cta ttt gat agt gac cta cct tcg gaa ctt aaa
     Asp Lys Leu Ser Leu Asp Gly Ser Leu Glu Phe

     cct gat caa aag gac ctg agg tct gct gta gac
 925 --+ --- --- --- +-- --- --- -+- --- --- --+
     gga cta gtt ttc ctg gac tcc aga cga cat ctg
     Pro Asp Gln Lys Asp Leu Arg Ser Ala Val Asp

     aag gct ata aat gct gat gtc act gga cag gcg
 958 --- --- --- +-- --- --- -+- --- --- --+ ---
     ttc cga tat tta cga cta cag tga cct gtc cgc
     Lys Ala Ile Asn Ala Asp Val Thr Gly Gln Ala

     gac aag ggg aga gac ttt ttt ggc agt ata cca
 991 --- --- +-- --- --- -+- --- --- --+ --- ---
     ctg ttc ccc tct ctg aaa aaa ccg tca tat ggt
     Asp Lys Gly Arg Asp Phe Phe Gly Ser Ile Pro

     gga aag gtg aaa aat gag acc aga cta agt gtg
1024 --- +-- --- --- -+- --- --- --+ --- --- ---
     cct ttc cac ttt tta ctc tgg tct gat tca cac
     Gly Lys Val Lys Asn Glu Thr Arg Leu Ser Val

     cag ggg gct cta ttg gag ctt gaa aat tta aaa
1057 +-- --- --- -+- --- --- --+ --- --- --- +--
     gtc ccc cga gat aac ctc gaa ctt tta aat ttt
     Gln Gly Ala Leu Leu Glu Leu Glu Asn Leu Lys

     acc cgg atc tta agt gtg act aga gac ctt cct
1090 --- --- -+- --- --- --+ --- --- --- +-- ---
     tgg gcc tag aat tca cac tga tct ctg gaa gga
     Thr Arg Ile Leu Ser Val Thr Arg Asp Leu Pro

     ctt aat gtg ttc aac act acc tca gca tca cta
1123 --- -+- --- --- --+ --- --- --- +-- --- ---
     gaa tta cac aag ttg tga tgg agt cgt agt gat
     Leu Asn Val Phe Asn Thr Thr Ser Ala Ser Leu
     acg gac atg cag aaa tcg atc aaa ggt ttt gct
1156 -+- --- --- --+ --- --- --- +-- --- --- -+-
     tgc ctg tac gtc ttt agc tag ttt cca aaa cga
     Thr Asp Met Gln Lys Ser Ile Lys Gly Phe Ala

     cct tat ata gag cga tca agc caa atc agc cgg
1189 --- --- --+ --- --- --- +-- --- --- -+- ---
     gga ata tat ctc gct agt tcg gtt tag tcg gcc
     Pro Tyr Ile Glu Arg Ser Ser Gln Ile Ser Arg

     gat gtt gga ttg gtc atg agc tgt ctg att ttg
1222 --- --+ --- --- --- +-- --- --- -+- --- ---
     cta caa cct aac cag tac tcg aca gac taa aac
     Asp Val Gly Leu Val Met Ser Cys Leu Ile Leu

     ctg gtg gtg atc tgt aac ttt ctg ggt ctg ctg
1255 --+ --- --- --- +-- --- --- -+- --- --- --+
     gac cac cac tag aca ttg aaa gac cca gac gac
     Leu Val Val Ile Cys Asn Phe Leu Gly Leu Leu

     ctg ggc ata gct gga cta aat cct aaa gac aat
1288 --- --- --- +-- --- --- -+- --- --- --+ ---
     gac ccg tat cga cct gat tta gga ttt ctg tta
     Leu Gly Ile Ala Gly Leu Asn Pro Lys Asp Asn

     ccc tct gaa cgt tct ggc aca tct aac tgt gga
1321 --- --- +-- --- --- -+- --- --- --+ --- ---
     ggg aga ctt gca aga ccg tgt aga ttg aca cct
     Pro Ser Glu Arg Ser Gly Thr Ser Asn Cys Gly

     gga ata ttt ttc atg gca gga gta ggt ttc agc
1354 --- +-- --- --- -+- --- --- --+ --- --- ---
     cct tat aaa aag tac cgt cct cat cca aag tcg
     Gly Ile Phe Phe Met Ala Gly Val Gly Phe Ser

     ttc ctg gtc tcc tgg atc ttc atg tta gta gtg
1387 +-- --- --- -+- --- --- --+ --- --- --- +--
     aag gac cag agg acc tag aag tac aat cat cac
     Phe Leu Val Ser Trp Ile Phe Met Leu Val Val

     ttg ata ctt ttc ata gtg gga gga aat acc tat
1420 --- --- -+- --- --- --+ --- --- --- +-- ---
     aac tat gaa aag tat cac cct cct tta tgg ata
     Leu Ile Leu Phe Ile Val Gly Gly Asn Thr Tyr
     acc ctg atc tgt aaa gcc atg gca aac caa gga
1453 --- -+- --- --- --+ --- --- --- +-- --- ---
     tgg gac tag aca ttt cgg tac cgt ttg gtt cct
     Thr Leu Ile Cys Lys Ala Met Ala Asn Gln Gly

     act cat cca gct tat gac act cca ggg ctg att
1486 -+- --- --- --+ --- --- --- +-- --- --- -+-
     tga gta ggt cga ata ctg tga ggt ccc gac taa
     Thr His Pro Ala Tyr Asp Thr Pro Gly Leu Ile

     cca ggt ctg aac ctg tcc acg gtc ctg aat ttg
1519 --- --- --+ --- --- --- +-- --- --- -+- ---
     ggt cca gac ttg gac agg tgc cag gac tta aac
     Pro Gly Leu Asn Leu Ser Thr Val Leu Asn Leu
                  ^
     aaa aca aac ctg aat att ata aat gtg tac agt
1552 --- --+ --- --- --- +-- --- --- -+- --- ---
     ttt tgt ttg gac tta taa tat tta cac atg tca
     Lys Thr Asn Leu Asn Ile Ile Asn Val Tyr Ser

     gac tgt cag aag aat atg ccg ttg tgg acc aca
1585 --+ --- --- --- +-- --- --- -+- --- --- --+
     ctg aca gtc ttc tta tac ggc aac acc tgg tgt
     Asp Cys Gln Lys Asn Met Pro Leu Trp Thr Thr

     ttt cat ctg aat gag atc ttc gat cta aat agt
1618 --- --- --- +-- --- --- -+- --- --- --+ ---
     aaa gta gac tta ctc tag aag cta gat tta tca
     Phe His Leu Asn Glu Ile Phe Asp Leu Asn Ser

     aaa tta gat gtt tca cag tac act aat gag att
1651 --- --- +-- --- --- -+- --- --- --+ --- ---
     ttt aat cta caa agt gtc atg tga tta ctc taa
     Lys Leu Asp Val Ser Gln Tyr Thr Asn Glu Ile

     gat cag acc ttt aat ggg tta caa atc aac ata
1684 --- +-- --- --- -+- --- --- --+ --- --- ---
     cta gtc tgg aaa tta ccc aat gtt tag ttg tat
     Asp Gln Thr Phe Asn Gly Leu Gln Ile Asn Ile

     gca aac atc acc atc ctc agt cca gag gta aag
1717 +-- --- --- -+- --- --- --+ --- --- --- +--
     cgt ttg tag tgg tag gag tca ggt ctc cat ttc
     Ala Asn Ile Thr Ile Leu Ser Pro Glu Val Lys
     tcc cag cta aac aac ttc tcc agc agg acc agc
1750 --- --- -+- --- --- --+ --- --- --- +-- ---
     agg gtc gat ttg ttg aag agg tcg tcc tgg tcg
     Ser Gln Leu Asn Asn Phe Ser Ser Arg Thr Ser

     agt atg aac ttc agc aac att ata cat cag att
1783 --- -+- --- --- --+ --- --- --- +-- --- ---
     tca tac ttg aag tcg ttg taa tat gta gtc taa
     Ser Met Asn Phe Ser Asn Ile Ile His Gln Ile
              ▲
     aat gac ttg tca ggg aat gat ctg agc tca gta
1816 -+- --- --- --+ --- --- --- +-- --- --- -+-
     tta ctg aac agt ccc tta cta gac tcg agt cat
     Asn Asp Leu Ser Gly Asn Asp Leu Ser Ser Val

1849 gca gaa agt ctg gat gtc ttg gct ggc aaa cag
     --- --- --+ --- --- --- +-- --- --- -+- ---
     cgt ctt tca gac cta cag aac cga ccg ttt gtc
     Ala Glu Ser Leu Asp Val Leu Ala Gly Lys Gln

1882 agt aac caa tct aaa aaa gat gaa ctg cac ggg
     --- --+ --- --- --- +-- --- --- -+- --- ---
     tca ttg gtt aga ttt ttt cta ctt gac gtg ccc
     Ser Asn Gln Ser Lys Lys Asp Glu Leu His Gly

     gaa gca aaa gat ctg cgt gac atc cag act gac
1915 --+ --- --- --- +-- --- --- -+- --- --- --+
     ctt cgt ttt cta gac gca ctg tag gtc tga ctg
     Glu Ala Lys Asp Leu Arg Asp Ile Gln Thr Asp

     gtc atg tct aac att atg cct ttg ctg aag gaa
1948 --- --- --- +-- --- --- -+- --- --- --+ ---
     cag tac aga ttg taa tac gga aac gac ttc ctt
     Val Met Ser Asn Ile Met Pro Leu Leu Lys Glu

     ctg aac agc act gtt aag aat ctc agt gaa gaa
1981 --- --- +-- --- --- -+- --- --- --+ --- ---
     gac ttg tcg tga caa ttc tta gag tca ctt ctt
     Leu Asn Ser Thr Val Lys Asn Leu Ser Glu Glu
          ▲
     gca tca caa atc act gca gct atg gaa aat gtt
2014 --- +-- --- --- -+- --- --- --+ --- --- ---
     cgt agt gtt tag tga cgt cga tac ctt tta caa
     Ala Ser Gln Ile Thr Ala Ala Met Glu Asn Val
     ttc aaa gaa gtt ggc tat gct cag gac gtc ctg
2047 +-- --- --- -+- --- --- --+ --- --- --- +--
     aag ttt ctt caa ccg ata cga gtc ctg cag gac
     Phe Lys Glu Val Gly Tyr Ala Gln Asp Val Leu

     aac tac aac att tca aaa ata gtg caa act gaa
2080 --- --- -+- --- --- --+ --- --- --- +-- ---
     ttg atg ttg taa agt ttt tat cac gtt tga ctt
     Asn Tyr Asn Ile Ser Lys Ile Val Gln Thr Glu
              ^

2113 agc agg gca ttc gta gac tgt cag ata aaa atc
     --- -+- --- --- --+ --- --- --- +-- --- ---
     tcg tcc cgt aag cat ctg aca gtc tat ttt tag
     Ser Arg Ala Phe Val Asp Cys Gln Ile Lys Ile

2146 ttt cag aca ttt ctt tac tgg gca aat caa acg
     -+- --- --- --+ --- --- --- +-- --- --- -+-
     aaa gtc tgt aaa gaa atg acc cgt tta gtt tgc
     Phe Gln Thr Phe Leu Tyr Trp Ala Asn Gln Thr
                                      ^

     atc aca gag aag gtg ggt cgt tgt ggg cct gca
2179 --- --- --+ --- --- --- +-- --- --- -+- ---
     tag tgt ctc ttc cac cca gca aca ccc gga cgt
     Ile Thr Glu Lys Val Gly Arg Cys Gly Pro Ala
                                  ◆       ◆

     gct gca gca gta gac aga tca gag gag ctc gtc
2212 --- --+ --- --- --- +-- --- --- -+- --- ---
     cga cgt cgt cat ctg tct agt ctc ctc gag cag
     Ala Ala Ala Val Asp Arg Ser Glu Glu Leu Val

     tgt aaa cac ttg gta gaa tct ttg aat gcc ttt
2245 --+ --- --- --- +-- --- --- -+- --- --- --+
     aca ttt gtg aac cat ctt aga aac tta cgg aaa
     Cys Lys His Leu Val Glu Ser Leu Asn Ala Phe
      ◆                       ◆

     tgg ttg agt ttg ggc tgg tgt atg atg ttc ctg
2278 --- --- --- +-- --- --- -+- --- --- --+ ---
     acc aac tca aac ccg acc aca tac tac aag gac
     Trp Leu Ser Leu Gly Trp Cys Met Met Phe Leu
      ◆           ◆                   ◆   ◆

     atc ccc agc atc att ttc tca gtt aaa ctg gcc
2311 --- --- +-- --- --- -+- --- --- --+ --- ---
     tag ggg tcg tag taa aag agt caa ttt gac cgg
     Ile Pro Ser Ile Ile Phe Ser Val Lys Leu Ala
      ◆
     aag tac tac cgc agg atg aag tat tca gat gct
2344 --- +-- --- --- -+- --- --- --+ --- --- ---
     ttc atg atg gcg tcc tac ttc ata agt cta cga
     Lys Tyr Tyr Arg Arg Met Lys Tyr Ser Asp Ala

     tat gag aac aat aac ttc atg atg aat cct ttc
2377 +-- --- --- -+- --- --- --+ --- --- --- +--
     ata ctc ttg tta ttg aag tac tac tta gga aag
     Tyr Glu Asn Asn Asn Phe Met Met Asn Pro Phe

     cca aag gcc acc tca aac ctg aat tat tgg gaa
2410 --- --- -+- --- --- --+ --- --- --- +-- ---
     ggt ttc cgg tgg agt ttg gac tta ata acc ctt
     Pro Lys Ala Thr Ser Asn Leu Asn Tyr Trp Glu

     atg gca aac aac cac att tta cat aag cac ata
2443 --- -+- --- --- --+ --- --- --- +-- --- ---
     tac cgt ttg ttg gtg taa aat gta ttc gtg tat
     Met Ala Asn Asn His Ile Leu His Lys His Ile

     aat taa
2476 -+- ---
     tta att
     Asn ***
```

Figure 5.1 Goldfish prominin ORF nucleotide and amino acid sequence 5' to 3'. The predicted signal peptide cleavage site is denoted by an arrow (↘). Predicted transmembrane domains are underlined with a solid line. Predicted N-glycosylation sites are indicated by an upward pointed arrow (^). The conserved cysteine rich region is *italicized* and the cysteine restudies are denoted by asterisks (*). The prominin family signature sequence is highlighted in grey with key residues marked with diamonds (◆).

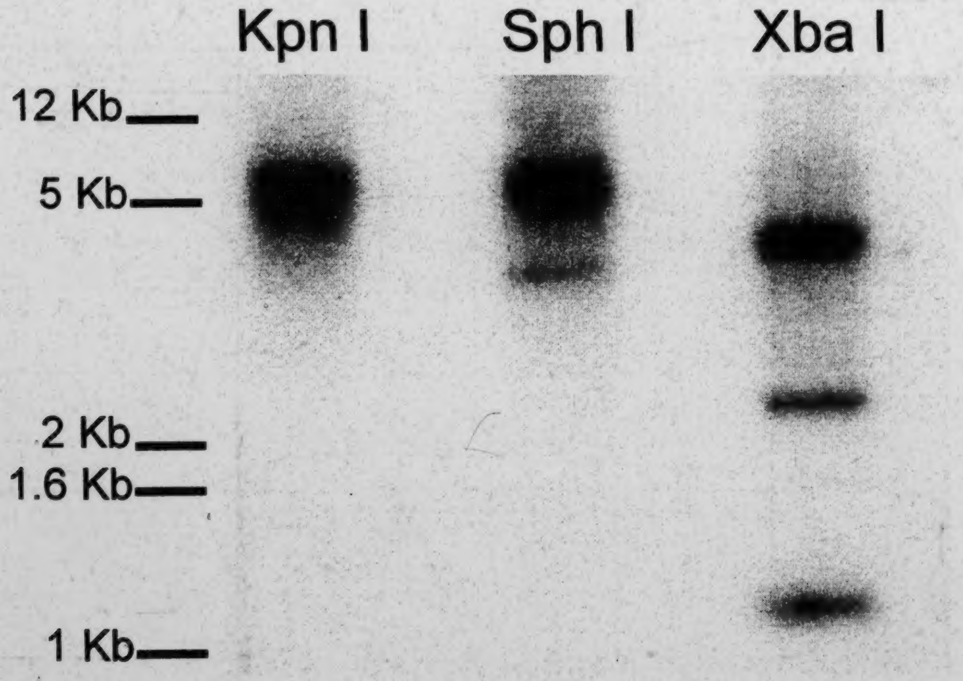
Kpn I
Sph I
Xba I
12 Kb
5 Kb
2 Kb
1.6 Kb
1 Kb

Figure 5.2 Southern blot of goldfish genomic DNA probed with a portion of the goldfish prominin sequence. Digestions were done using the enzymes Kpn I, Sph I and Xba I for 24hrs. Washes were preformed under moderate stringency. A size standard is shown on the left.

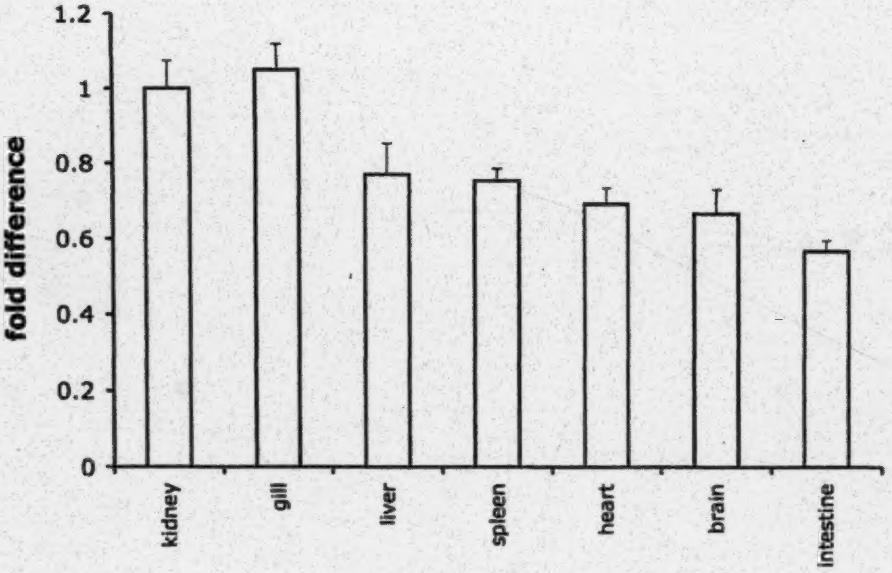

Figure 5.3 Real time PCR analysis showing tissue expression of goldfish prominin relative to the endogenous control β-actin for kidney, gill, liver, spleen, heart, brain and intestine. Relative expression is reported as the fold difference as compared to the chosen calibrator tissue (kidney). For each tissue total RNA was obtained from 5 individual fish.

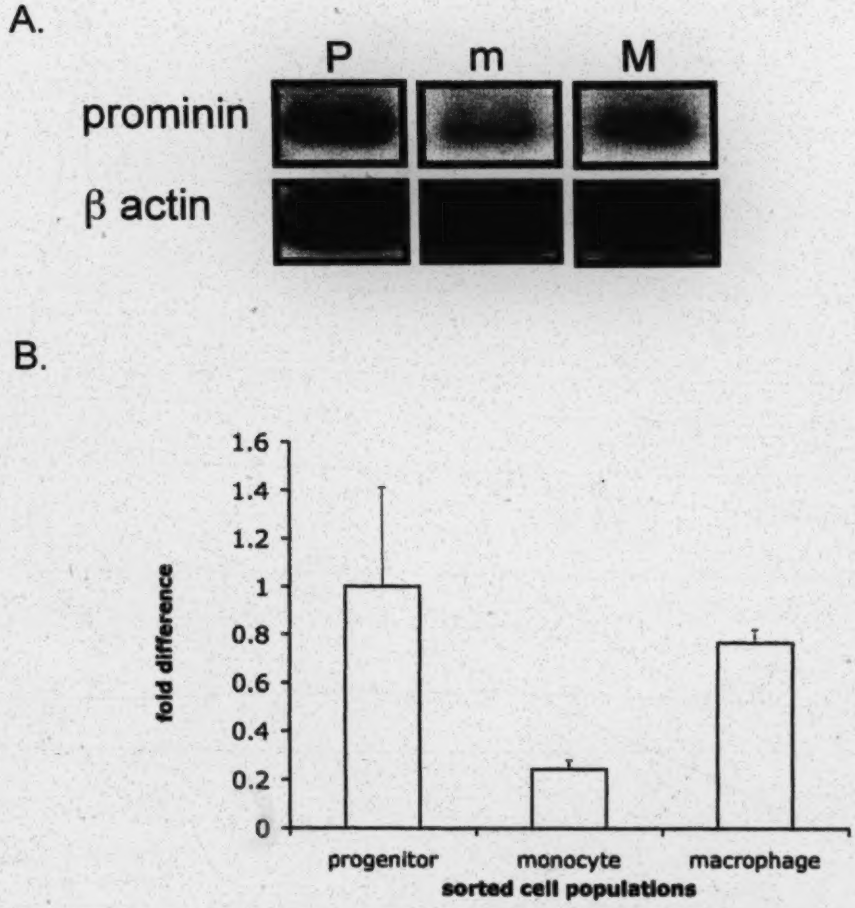

Figure 5.4 PCR analysis of goldfish prominin mRNA expression in sorted *in vitro*-derived macrophage subpopulations. A. RT-PCR analysis comparing expression of goldfish prominin to a β-actin loading control in sorted progenitor cells (P), monocytes (m) and mature macrophages (M). B. Real-time PCR analysis of prominin expression relative to the endogenous control β-actin . Relative expression is reported as the fold difference as compared to the chosen calibrator (progenitor subpopulation). Total RNA was obtained from 6 fish.

	Goldfish DQ233501	D. rerio XP 688810	T.nigrovirids CAF91648.1	H. sapiens Type 1 AAS19705	M.musculus Type 1 AAH28286	H. sapiens Type 2 NP 545934
Goldfish	**100**					
D. rerio	**29**	**100**				
T.nigroviridis	**38**	**28**	**100**			
H. sapiens Type 1	**28**	**42**	**25**	**100**		
M. musculus Type 1	**27**	**41**	**24**	**59**	**100**	
H. sapiens Type 2	**28**	**28**	**27**	**26**	**27**	**100**

Table 5.1 Percent amino acid identity of alignment of goldfish prominin with reprosentitive vertebrate prominins. The genbank accession number for each of the compaired sequences is reported in the first row.

CHAPTER 6

GENERAL DISCUSSION

Macrophages are fascinating subjects for study since they are central cells of the innate immune response. One of the integral components of innate immunity is the process called inflammation. Macrophages participate in the inflammatory responses of vertebrates at various levels: upon activation they have the ability to locate at the inflammatory sites through the process known as chemotaxis and upon arrival to these sites they are capable of mounting potent antimicrobial responses that ensure the destruction of the invading pathogens [1, 155-156]. These responses have been well characterized and include phagocytosis, the production of reactive oxygen and nitrogen intermediates (which are highly toxic for the invading pathogens) and production of pro-inflammatory cytokines whose role is the regulation of the inflammatory response [155, 157].

The competent inflammatory response requires the continuous production of new effector cells. The source of these new cells is the hematopoietic progenitor populations located in the bone marrow of mammals or the kidney of fish [140, 158]. In mammals, the members of the prominin family are markers of hematopoietic progenitor populations and the characterization of this molecule in goldfish will enable the identification of hematopoietic machinery required for induction of teleost inflammatory responses.

Tumor necrosis factor alpha (TNFα), a pleiotropic cytokine produced by macrophages, has a broad regulatory role in inflammation. Since the inflammatory response is of central importance in fish host defense, the characterization of this cytokine in goldfish is critical to an understanding of goldfish macrophage biology.

Within the context of inflammation, TNFα is critical for the initiation of the appropriate antimicrobial mechanisms that act to halt or contain infection. Thus, many of the events viewed as necessary for the successful early immune response of fish can be linked to TNFα. The open reading frame of goldfish TNFα-2 contained all of the hallmark regions identified for other fish TNFα sequences, including a pair of conserved cysteine residues and the TNF family signature [91-94]. I also identified a partial sequence of the TNFα-1 isoform. With the finding of multiple isoforms of TNFα in the goldfish, I investigated the differential expression of these two isoforms at the level of transcription. The expression of the TNF isoforms in tissues of the goldfish was different, such that TNFα-1 was more constitutively expressed than TNFα-2. Furthermore, in *in vitro*-derived primary macrophages, TNFα-1 was expressed both constitutively and in response to activation, while TNFα-2 was primarily produced in response to activation.

To further characterize the role of goldfish TNFα upon macrophage activation, I produced a recombinant molecule based on the mature portion of TNFα-2. The biological activities of the recombinant protein that I examined were comparable to those described for the native TNFα. These activities included the induction of chemotactic response, phagocytosis, the production of nitric oxide and up-regulation of pro-inflammatory cytokine production.

In the second part of this thesis I have presented evidence for the presence of a prominin molecule in goldfish. Although the overall sequence was not highly conserved amongst the prominins, the characteristic pentaspan membrane organization as well as the prominin family signature clearly placed the goldfish sequence amongst this unique group of molecules. As is the case for its mammalian counterparts, goldfish prominin was broadly expressed in tissues and highly expressed in the macrophage progenitor subpopulation, suggesting that it may be used as a hematopoietic marker in fish.

Throughout this thesis I have referred to the goldfish tumor necrosis factor sequences as a TNF "alpha"; it should be noted that this nomenclature comes with a caveat because of the unresolved question regarding the existence of TNFβ in the teleosts. The absence of a TNFβ gene in fish, despite concerted efforts to identify it, has lead some to speculation that teleost TNF is the ancestral gene that gave rise to TNFα and β through duplication later in evolution. Recently, researchers have identified TNF genes from fish that are not similar to TNFα but there is still not enough evidence to support the identity of some of these as TNFβ-like. In fact the combined evidence seem to suggest that these are lymphotoxin beta homologs [102-103]. However, even in the absence of fish TNFβ, goldfish TNF and its counterparts amongst the teleosts are most structurally similar to mammalian TNFα. This is supported by the conserved structures of the molecule such as the trans-membrane domain region, conserved cysteines and the TNF signature sequence [91-94].

Because TNFα is involved in the regulation of so many different aspects of the inflammatory/antimicrobial response, the presence of multiple isoforms of goldfish TNF is intriguing because it suggests diverse, isotype-specific functions for this molecule. The presence of multiple TNFα genes is a state unique to only some teleost species such as goldfish, carp and trout [82, 92]. Japanese flounder and catfish for example only possess a single TNFα encoding gene, as is the case for humans and mice [91, 94]. The multiple isoforms of goldfish, carp and trout are thought to have arisen following a genome duplication event, an occurrence that represents a global and non-specific reorganization of the organism's genetic parameters. However, the differential expression of the TNFα isoforms suggests that there are selective pressures acting on TNFα that are leading to a division of roles. Supporting such a conclusion is the evidence from studies of a number of different mammalian TNFα isoforms, produced as a result of post-transcriptional/translational modifications [87, 159]. The functional significance of TNFα isoforms is not fully understood even in the mammalian systems. The production of TNFα isoforms by mouse macrophages was differentially regulated depending on whether the cells were stimulated with LPS or cytokines such granulocyte-macrophage-colony-stimulating-factor (GM-CSF), which seems to indicate a tailoring of the TNFα response in anticipation of a specific functional requirement [87]. Certain alternatively cleaved isoforms of TNFα may have special significance in host defense against intracellular pathogens as isoforms differing in size by only1 kD were widely different in their ability to induce cytotoxic responses [159].

The sequence identity between goldfish TNFα-1 and 2 was high (~83%), suggesting a possibility of significant functional overlap between isoforms. A study comparing the activity of the two isoforms of trout TNFα found that their ability to induce phagocytosis and chemotaxis was similar [92]. Regardless of whether TNFα isoforms are the result of gene duplication as in fish or the type of post-transcriptional/translational modifications observed in mammals, the presence of such diversity suggests that the pleiotropic TNFα response is to some degree specialized at the level of the TNFα ligand.

As indicated in chapter 2 the vast majority of the macrophages involved in host defense must be recruited to the inflammatory site. This process begins with adhesion to the vascular epithelium followed by chemotaxis to the lesion site. TNFα has been shown to up-regulate the expression of adhesion molecules such as ICAM on the surface of epithelial cells as well as induce the production of chemokines which attract cells to the sites of infection [157, 160-161]. TNFα knockout mice were shown to be highly susceptible to *Mycobacterium* infections in part because chemokine production and therefore the recruitment of leukocytes toward inflammatory sites was delayed. Similarly, knockout mice are unable to generate a granulomatous response which is an important barrier for dissemination of the bacteria in the tissues [157, 161].

TNFα has also an important regulatory role in the directional migration of fish leukocytes both through the up regulation of other chemoattractive molecules such as IL-8 [98] as well through direct stimulus. For example, intraperitoneal injection of both turbot and seabream recombinant TNFα was shown to mobilize leukocytes to the site of injection. Similarly, recombinant trout TNFα was shown to induce chemotaxis of macrophages *in vitro* [98], and I have demonstrated that goldfish recombinant TNFα also induced chemotaxis of *in vitro*-derived kidney macrophages.

The importance of phagocytosis in the overall antimicrobial response of macrophages cannot be overstated since vast majority of antimicrobial responses require competent phagocytic cells. Once ingested, microbes within the phagosome/ phagolysosomes are assaulted by a barrage of antimicrobial proteins and peptides [156]. Alternatively, pathogens within macrophages can be deprived of essential nutrients such as iron or tryptophan, an effective way in preventing their replication within the cells [162].

The activation of macrophages and the initiation of the phagocytic response is often associated with a marked increase in the oxygen consumption, resulting in the production of highly toxic reactive oxygen species, a process known as the "respiratory burst" [155]. The importance of reactive oxygen production to the innate immune defense is exemplified in the case of individuals with chronic granulomatous disease (CGD).

CGD individuals have inhibitory mutations in the enzymatic machinery controlling this response, resulting in an increased susceptibility to recurrent bacterial infections and a reduced life expectancy [155]. TNFα has been shown to prime the respiratory burst response in both mammals and fish [95, 163]. A role for the respiratory burst response has also been suggested in studies of fish diseases, including the protective response induced by vaccination against protozoan parasites and the response of leukocytes to intraperitoneal injection of pathogenic bacteria [164-165].

Related to the production of reactive oxygen intermediates but under separate enzymatic control is the production of reactive nitrogen intermediates of which nitric oxide is the principle product. The nitric oxide (NO) response is a very potent antimicrobial response of fish macrophages. Nitric oxide production has been shown to enhance resistance to diseases such as malaria and tuberculosis and conversely, reduction in NO is linked to increased susceptibility to these infections [155]. TNFα was shown to up-regulate the NO response of macrophages, and the central role for TNFα in this antimicrobial response was exemplified in mycobacterial infections where TNF deficient mice exhibited reduced NO production and an inability to control this infection [157]. Increased nitric oxide response was reported for fish vaccinated against the pathogenic bacteria *Photobacterium damselae* and *Aeromonas hydrophila* and was related to enhanced protection [70, 84]. Inhibition of the NO response in fish has been shown significantly increase the susceptibility to bacterial infection in both seabream and catfish [70, 84]. It should be noted, however, that a strong nitric oxide response may not be beneficial in all cases. Studies of the susceptibility of different strains of carp to blood-borne trypanosome *Trypanoplasma borreli,* demonstrated that elevated NO production resulted in the inability of susceptible carp strains to control the infection. This was linked to a polymorphism in the TNFα genes [82]. In my research, I examined the effect of a related parasite (*Trypanosoma danilewskyi*) on the NO response of goldfish macrophages. My results did not demonstrate up regulation of the NO production in infected goldfish, nor increased expression of TNFα, suggesting that the induction of the NO response in fish may be host an/or parasite-specific. Studies in carp suggested that TNFα had a regulatory role in induction of the NO response in fish [82]. My results confirm these findings in that recombinant TNFα was capable of inducing a potent NO response in goldfish macrophages.

In the context of the competent immune system, development and proliferation of immune cells is essential component in host defense against pathogens. The fish developmental models, (primarily the zebrafish model), are fast gaining prominence as powerful molecular systems, however, the reliance on morphological assessment of developmental pathways is still significant. In the absence of specific molecular reagents (such as monoclonal Abs, etc..) some researchers have adopted an approach that combines morphological and molecular analysis. Traver et al. [166] used flow cytometry to separate blood cell populations of zebrafish and confirmed the identities of the sorted cell subpopulations using lineage specific markers. This system allowed these researchers to gain new insights into the nature of several known bloodless zebrafish mutants [166]. Our lab has developed a similar flow cytometry based method to analyze the development of goldfish macrophages *in vitro* and has generated a number of key markers and regulators of macrophage hematopoiesis [135, 167-168]

The identification of prominin expression in a specific hematopoietic cell type may indicate that an AC133 like epitope that marks stem cells can be identified in fish. However, beyond its utility as a marker, prominin may yet have an interesting and active role in the regulation of goldfish macrophage development. Recent work by Marzesco et al. [153] reported intriguing evidence of the release of prominin carrying extracellular membrane particles from developing mouse neuroepithelial cells. Results such as these raise questions about the importance of cell-scale influences of hematopoiesis, including cell morphology, polarity and microenvironment, scale issues that may well be model species specific.

Future directions

The role of multiple isoforms of fish TNFα has not been fully examined although there is preliminary evidence from carp [93], trout [92] and now goldfish that this is an aspect of fish TNFα biology that would warrant further investigation. In light of the mammalian literature, an in-depth study of TNFα isoforms in fish should include an investigation of the presence of post transcriptional/translational modification of fish TNFα.

Although some functional differences have been observed for the trout TNFα isoforms, on the whole their function was reported to be quite similar [92]. However, the production of a recombinant protein in a prokaryotic system (as were the trout isoforms) may not be the optimal way to examine functional differences between isoforms as more subtle differences in arrangement would likely be absent in recombinant proteins expressed using a prokaryote expression system. Therefore, despite the demonstrated biological activity of the goldfish TNFα-2, production of the goldfish TNFα-1 and 2 using eukaryotic systems, may allow further analyses of the importance of modifications such as glycosylation in the function of fish TNFs. Furthermore, the creation of an effective antibody against native fish TNFα would be invaluable towards an investigation of mRNA and protein level alterations to the TNFα molecules of fish.

The current recombinant TNFα-2 molecule studies are going ahead to examine additional biological roles for this molecule in teleosts including the induction of the respiratory burst and proliferation of macrophages *in vitro*, as well as potential biological effects of the teleost TNFs *in vivo*.

The expression of a prominin molecule on the progenitor cells isolated from goldfish kidneys raises the possibility that an antibody against the AC133 epitope could be developed for fish. The ability to select for fish progenitor or stem cells in this manner would constitute a major advance in our efforts to understand hematopoietic pathways of teleosts.

CHAPTER 7

LITURATURE CITED

[1] Nathan C. Neutrophils and immunity: challenges and opportunities. Nature Reviews Immunology. 2006; 6:173

[2] Imof BA, Aurrand-Lions M. Adhesion mechanisms regulating the migration of monocytes. Nature Reviews Immunology. 2004; 4:432

[3] Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, Olweus J, Kearney J, Buck DW. AC133, a Novel Marker for Human Hematopoietic Stem and Progenitor Cells. Blood. 1997; 90:5002

[4] Goetz FW, Planas JV, MacKenzie S. Tumor necrosis factors. Developmental and Comparative Immunology. 2004; 28:487

[5] Muller WA. Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. TRENDS in Immunology. 2003; 24:326

[6] Matsuyama T. Iida T. Tilapia mast cell lysates enhance neutrophil adhesion to cultured vascular endothelial cells. Fish and Shellfish Immunology. 2002; 13:243

[7] Huhtala M, Heino J, Casciari D, de Luise A, Johnson MS. Integrin evolution: Insights from ascidian and teleost fish genomes. Matrix Biology. 2005; 24:83

[8] Mulero V, Pelegrin P, Sepulcre MP, Munoz J, Meseguer J. A fish cell surface receptor defined by a mAb mediates leukocyte aggregation and deactivation. Developmental and Comparative Immunology. 2001; 25:619

[9] Ainsworth AJ, Ye Q, Xue L, Hebert P. Channel catfish, *Ictalurus punctaltus* Rafinesque, neutrophil adhesion to selected extracellular matrix proteins, lipopolysaccharide, and catfish serum. Devopmental and Comparative Immunology. 1996; 20:105

[10] Qian Y, Ainsworth AJ, Noya M. Identification of a beta 2 (CD18) molecule in a teleost species, *Ictalurus punctatus* Rafinesque. Developmental and Comparative Immunology. 1999; 23:571

[11] Reinhart B, DeWitte-Orr SJ, Van Es SJ, Bols NC, Lee LEJ. Cell adhesion characteristics of a monocytic cell line derived from rainbow trout, *Oncorhynchus mykiss.* Comparative Biochemistry and Physiology. 2006 in press

[12] Qian Y, Noya M, Ainsworth AJ. Molecular characterization and leukocyte distribution of a teleost β1 integrin molecule. Veterinary Immunology and Immunopathology. 2000; 76:61

[13] Nakao M, Fujiki K, Kondo M, Yano T. Detection of complement receptors on head kidney phagocytes of the common carp *Cyrinus carpio*. Fisheries Science. 2003; 69:929

[14] Goetz FW, Iliev DB, McCauley LAR, Liarte CQ, Tort LB, Planas JV, MacKenzie J. Analysis of genes isolated from lipopolysaccharide-stimulated rainbow trout (*Oncorhynchus mykiss*) macrophages. Molecular Immunology. 2004; 41:1199

[15] Laing KJ, Secombes CJ. Chemokines. Developmental and Comparative Immunology. 2004; 28:443

[16] Boshra H, Li J, Sunyer JO. Recent advances on the complement system of teleost fish. Fish and Shellfish Immunology. 2006; 20:236

[17] Najakshin AM, Mechetina LV, Alabyev BY, Taranin AV. Identification of an IL-8 homolog in lamprey (Lampetra fluviatilis): early evolutionary divergence of chemokines. European Journal of Immunology. 1999; 29:375

[18] Inoue Y, Haruta C, Usui K, Moritomo T, Nakanishi T. Molecular cloninf and sequencing of the banded dogfish (*Triakis scyllia*) interleukin-8 cDNA. Fish and Shellfish Immunology. 2003; 14:275

[19] Inoue Y, Endo M, Haruta C, Taniuchi T, Moritomo T, Nakanishi T. Molecular cloning and sequencing of the silver chimaera (*Chimaera phantasma*) interleukin-8 cDNA. Fish and Shellfish Immunology. 2003; 15:269

[20] Lee EY, Park HH, Kim YT, Choi TJ. Cloning and sequence analysis of the interleukin-8 gene from flounder (*Paralichthys olivaceous*). Gene. 2001; 274:237

[21] Laing KJ, Zou JJ, Wang T, Bols N, Hirono I, Aoki T, Secombes CJ. Identification and analysis of an interleukin 8-like molecule in rainbow trout *Oncorhynchus mykiss.* Developmental and Comparative Immunology. 2002; 26:433

[22] Baoprasertkul P, Peatman Eric, Chen L, He C, Kucuktas H, Li P, Simmons M, Liu Z. Sequence analysis an expression of a CXC chemokine in resistant and susceptible catfish after infection of *Edwardsiella ictalus*. Developmental and Comparative Immunology. 2004; 28:769

[23] Baoprasertkul P, He C, Peatman E, Zhang S, Li P, Liu Z. Constitutive expression of three novel catfish CXC chemokines: homeostatic chemokines in teleost fish. Molecular Immunology. 2005; 42:1355

[24] Huising MO, Stolte E, Flik G, Savelkoul HFJ, Verburg-van Kemenade BML. CXC chemokines and leukocytes chemotaxis in common carp (*Cyprinus carpio L.*). Developmental and Comparative Immunology. 2003; 27:875

[25] Dixon B, Shum B, Adams EJ, Magor KE, Hedrick RP, Muir DG, Parham P. CK-1, a putative chemokine of rainbow trout (*Oncorhynchus mykiss*). Immunological Reviews. 1998; 166:341

[26] Liu L, Fujiki K, Dixon B, Sundick RS. Cloning of a novel rainbow trout (*Oncorhynchus mykiss*) CC chemokine with a fractalkine-like stalk and a TNF decoy receptor using cDND fragments containing AU-rich elements. Cytokine. 2002; 17:71

[27] Fujiki K, Shin DH, Nakao M, Yano T. Molecular cloning of carp (*Cyprinus carpio*) CC chemokine, CXC chemokine receptors, allograft inflammatory factor-1, and natural killer cell enhancing factor by use of suppression subtractive hybridization. Immunogenetics. 1999; 49:909

[28] Kono T, Kusuda R, Kawahara E, Sakai M. The analysis of immune responses of a novel CC-chemokine gene from Japanese flounder *Paralichthys olivaceus*. Vaccine. 2003; 21:446

[29] Lally J, Al-Anouti F, Bols N, Dixon B. The functional characterization of CK-1, a putative CC chemokine from rainbow trout (*Oncorhynchus mykiss*). Fish and Shellfish Immunology. 2003; 15:411

[30] Newton RA, Raftos DA, Raison RL, Geczy CL. Chemotactic responses of hagfish (Vertebrata Agnatha) leucoytes. Developmental and Comparative Immunology. 1994; 18:295

[31] Boshra H, Peters R, Li J, Sunyer O. Production of recombinant C5a from rainbow trout (*Oncorhynchus mykiss*): roll in leukocyte chemotaxis and respiratory burst. Fish and Shellfish Immunology. 2004; 17:293

[32] Li J, Peters R, Lapatra S, Vazzana M, Sunyer JO. Anaphylatoxin-like molecules generated during complement activation induce a dramatic enhancement of Particle uptake in rainbow trout phagocytes. Developmental and Comparative Immunology. 2004; 28:1005

[33] Kato Y, Nakao M, Shimizu M, Wariishi H, Yano T. Purification and functional assessment of C3a, C4a and C5a of the common carp (*Cyprinus carpioi*) complement. Developmental and Comparative Immunology. 2004; 28:901

[34] Claire M, Holland H, Lambris JD. A functional C5a anaphylatoxin receptor in a teleost species. Journal of Immunology. 2004; 172:349

[35] Rotllant J, Parra D, Peters R, Boshra H, Sunyer JO. Generation, purification and functional characterization of three C3a anaphylatoxins in rainbow trout: Role in leukocyte chemotaxis and respiratory burst. Developmental and Comparative Immunology. 2004; 28:815

[36] Froystad MK, Rode M, Berg T, Gjoen T. A role for scavenger receptors in phagocytosis of protein-coated particles in rainbow trout head kidney macrophages. Developmental and Comparative Immunology. 1998; 22:533

[37] Jenkins JA, Ourth DD. Opsonic effect of the alternative complement pathway on channel catfish peripheral blood phagocytes. Veterinary Immunology and Immunopathology. 1993; 39:447

[38] Johnson E, Smith P. Attachment and phagocytosis by salmon macrophages of agrose beads coated with human C3b and C3bi. Developmental and Comparative Immunology. 1984; 8:623

[39] McKinney EC, Flajnik MF. IgM-mediated opsonization and cytotoxicity in shark. Journal of leukocyte Biology. 1997; 61:141

[40] Sakai DK. Opsonization by fish antibody and complement in the immune phagocytosis by peritoneal exudates cells isolated from salmonid fishes. Journal of Fish Diseases. 1984; 7:29

[41] O'Dowd AM, Ellis AE, Secombes CJ. Binding of immune complexes to Atlantic salmon peripheral blood leucocytes. Developmental and Comparative Immunology. 1998; 22:439

[42] Griffin BR. Opsonic effect of rainbow trout (*Salmo gairdneri*) antibody on phagocytosis of *Yersinia ruckeri* by trout leukocytes. Developmental and Comparative Immunology. 1983; 7:253

[43] Koumans-van Diepen JCE, van de Lisdonk MHM, Taverne-Thiele AJ, Verburg-van Kemenade BML, Rombout JHWM. Characterization of Immunoglobulin-binding leucocytes in carp (*Cyprinus carpio* L.). Developmental and Comparative Immunology. 1994; 18:45

[44] Haynes L, Fuller L, McKinney EC. Fc receptor for shark IgM. Developmental and Comparative Immunology. 1988; 12:561

[45] Fujiki K, Shin DH, Nakao M, Yano T. Molecular cloning and expression analysis of carp (*Cyprinus carpio*) interleukin-1β, high affinity immunoglobulin E Fc receptor γ subunit and serum amyloid A. Fish and Shellfish Immunology. 2000; 10:229

[46] Shen L, Stuge TB, Evenhuis JP, Bengten E, Wilson M, Chinchar VG, Clem LW, Miller NW. Channel catfish NK-like cells are armed with IgM via a putative FcμR. Developmental and Comparative Immunology. 2003; 27:699

[47] Engstad RE, Robertsen B. Recognition of yeast cell wall cglucan by Atlantic salmon (*Salmo salar* L.) macrophages. Developmental and Comparative Immunology. 1993; 17:319

[48] Dannevig BH, Lauve A, Press CM, Landsverk T. Receptor-mediated endocytosis and phagocytosis by rainbow trout head kidney sinusoidal cells. Fish and Shellfish Immunology. 1994; 4:3

[49] Leiro J, Ortega M, Estevez J, Ubeira FM, Sanmartin ML. The role of opsonization by antibody and complement in *in vitro* phagocytosis of microsporidian parasites by turbot spleen cells. Veterinary Immunology and Immunopathology. 1996; 51:201

[50] Iyer GYN, Islam MF, Quastel JH. Biochemical aspects of phagocytosis. Nature. 1961; 192:535

[51] Lambeth JD. NOX enzymes and the biology of reactive oxygen. Nature Reviews Immunology. 2004; 4:181

[52] Dang PM, Cross AR, Quinn MT, Babior BM. Assembly of the neutrophil respiratory burst oxidase: a direct interaction between p67PHOX and cytochrome b558 II. Proceedings of the National Academe of Science. 2002; 99:4262

[53] Reeves EP, Nagl M, Godovac-Zimmermann J, Segal AW. Reassessment of the microbicidal activity of reactive oxygen species and hypochlorous acid with reference to the phagocytic vacuole of the neutrophil granulocyte. Journal of Medical Microbiology. 2003; 52:643

[54] Cassatella MA, Della BV, Berton G, Rossi F. Activation by gamma interferon of human macrophage capacity to produce toxic oxygen molecules is accompanied by decreased Km of the superoxide-generating NADPH oxidase. Biochemical Biophysical Research Communications. 1985; 132:908

[55] Nathan CF, Murray HW, Wiebe ME, Rubin BY. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. Journal of Experimental Medicine. 1983; 158:670.

[56] Tsunawaki S, Nathan CF. Enzymatic basis of macrophage activation. Kinetic analysis of superoxide production in lysates of resident and activated mouse peritoneal macrophages and granulocytes. Journal of Biological Chemestry. 1984; 259:4305

[57] Newburger PE, Ezekowitz RA, Whitney C, Wright J, Orkin SH. Induction of phagocyte cytochrome b heavy chain gene expression by interferon gamma. Proceedings of the National Academe of Science. 1988; 85:5215

[58] Secombes CJ, Cross AR, Sharp GJ, Garcia R. NADPH oxidase-like activity in rainbow trout Oncorhynchus mykiss (Walbaum) macrophages. Developmental and Comparative Immunology. 1992;16:405

[59] Itou T, Iida T, Kawatsu H. Evidence for the existence of cytochrome b558 in fish neutrophils by polyclonal antipeptide antibody. Developmental and Comparative Immunology. 1998; 22:433

[60] Shiibashi T, Iida T, Itou T. Analysis of localization and function of the COOH-terminal corresponding site of cytochrome b558 in fish neutrophils. Developmental and Comparative Immunology. 1999; 23:213

[61] Shiibashi T, Iida T. NADPH and NADH serve as electron donor for the superoxide-generating enzyme in tilapia (Oreochromis niloticus) neutrophils. Developmental and Comparative Immunology. 2001; 25:461

[62] Inoue Y, Suenaga Y, Yoshiura Y, Moritomo T, Ototake M, Nakanishi T. Molecular cloning and sequencing of Japanese pufferfish (Takifugu rubripes) NADPH oxidase cDNAs. Developmental and Comparative Immunology. 2004; 28: 911

[63] Neumann NF, Fagan D, Belosevic M. Macrophage activating factor(s) secreted by mitogen stimulated goldfish kidney leukocytes synergize with bacterial lipopolysaccharide to induce nitric oxide production in teleost macrophages. Developmental and Comparative Immunology.1995; 22:433

[64] Neumann NF, Belosevic M. Deactivation of primed respiratory burst response of goldfish macrophages by leukocyte-derived macrophage activating factor(s). Developmental and Comparative Immunology. 1996; 20:427

[65] Stafford J, Neumann NF, Belosevic M. Inhibition of macrophage activity by mitogen-induced goldfish leukocyte deactivating factor. Developmental and Comparative Immunology. 1999; 23:585

[66] Graham S, Secombes CJ. Cellular requirements for lymphokine secretion by rainbow trout Salmo gairdneri leukocytes. Developmental and Comparative Immunology. 1990; 14:59

[67] Novoa B, Figueras A, Ashton I, Secombes CJ. *In vitro* studies on the regulation of reainbow trout (*Oncorhynchus mykiss*) macrophage respiratory burst activity. Developmental and Comparative Immunology. 1996; 20:207

[68] Mulero V, Meseguer J. Functional characterization of a macrophage activating factor produced by leukocytes of gilthead seabream (*Sparus aurata L.*). Fish and Shellfish Immunology. 1998; 8:143

[69] Neumann NF, Barreda DR, Belosevic M. Generation and functional analysis of distinct macrophage sub-populations from goldfish (*Carassius auratus L.)* kidney leukocyte cultures. Fish and Shellfish Immunology. 2000;10:1

[70] .Yin Z, Lam TJ, Sin YM. Cytokine-mediated antimicrobial immune response of catfish, Clarias gariepinus, as a defense against *Aeromonas hydrophila*. Fish and Shellfish Immunology. 1997; 7:93

[71] Milev-Milovanovic I, Long S, Wilson M, Bengten E, Miller NW, Chinchar VG. Identification and expression analysis of interferon gamma genes in channel catfish. Immunogenetics. 2006; 58:70

[72] Zou J, Yoshiura Y, Dijkstra JM, Sakai M, Ototake M, Secombes CJ. Identification of an interferon gamma homologue in Fugu, *Takifugu rubripes*. Fish and Shellfish Immunology. 2004; 17:403

[73] Igawa D, Sakai M, Savan R. An unexpected discovery of two interferon gamma-like genes along with interleukin (IL)-22 and -26 from teleost: IL-22 and -26 genes have been described for the first tine outside mammals. Molecular Immunology. 2006; 43:999

[74] Bogdan C. Nitric Oxide and the Immune Response. Nature Immunology. 2001; 2:907

[75] Laing KJ, Grabowski PS, Belosevic M, Secombes CJ. A partial sequence for nitric oxide synthase from a goldfish (Carassius auratus) macrophage cell line. Immunology and Cell Biology. 1996; 74:374

[76] Laing K, Hardie LJ, Aartsen W, Grabowski PS, Secombes CJ. Expression of an inducable nitric oxide synthase gene in rainbow trout Oncorhynchus mykiss. Developmental and Comparative Immunology. 1999; 23:71

[77] Wang R, Belosevic M. The *in vitro* effects of estradiol and cortisol on the function of a long-term goldfish macrophage cell line. Developmental and Comparative Immunology. 1995; 19:327

[78] Wang R, Neumann NF, Shen Q, Belosevic M. Establishment and characterization of a macrophage cell line from the goldfish. Fish and Shellfish Immunology. 1995; 5:329

[79] Bose M, Farnia P. Proinflammatory cytokines can significantly induce human mononuclear phagocytes to produce nitric oxide by a cell maturation-dependent process. Immmunology Letters. 1995; 48:59

[80] Martin JH, Edwards SW. Changes in mechanisms of monocyte/macrophage-mediated cytotoxicity during culture. Reactive oxygen intermediates are involved in monocyte-mediated cytotoxicity, whereas reactive nitrogen intermediates are employed by macrophages in tumor cell killing. Journal of Immunology. 1993; 150:3478

[81] Tafalla C, Novoa B. Requirements for nitric oxide production by turbot (Scophthalmus maximus) head kidney macrophages. Developmental and Comparative Immunology. 2000; 24:623

[82] Saeij JPJ, Stet RJM, de Vries BJ, van Muiswinkel WB, Wiegertjes GF. Molecular and functional characterization of carp TNF: a link between TNF polymorphism and trypanotolerance? Developmental and Comparative Immunology. 2003; 27:29

[83] Stafford JL, Wilson EC, Belosevic M. Recombinant transferrin induces nitric oxide response in goldfish and murine macrophages. Fish and Shellfish Immunology. 2004; 17:171

[84] Acosta F, Real F, Ellis AE, Tabraue C, Padilla D, Ruiz DE, Galarreta CM. Influence of vaccination on the nitric oxide response of gilthead seabream following infection with *Photobacterium damselae* subsp. piscicida. Fish and Shellfish Immunology. 2005; 18:31

[85] MacEwan DJ. TNF ligands and receptors - a matter of life and death. British Journal of Pharmacology. 2002; 135:855

[86] Schook LB, Albrecht H, Gallay P, Jongeneel CV. Cytokine regulation of TNF-α mRNA and protein production by unprimed macrophages from C57Bl/6 and NZW mice. Journal of Leukocyte Biology. 1994; 56:514

[87] Branch DR, Guilbert LJ. Differential expression of tumor necrosis factor-α isoforms from lipopolysaccharide- and cytokine-stimulated mouse macrophages. International Journal of Biochemistry and Cell Biology. 1996; 28:949

[88] Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilites: integrating mammalian biology. Cell. 2001; 104:487

[89] Pfeffer K, Biological functions of tumor necrosis factor cytokines and their receptors. Cytokine and Growth Factor Review. 2003; 14:185

[90] Quin QW, Ototake M, Noguchi K, Soma GI, Yokomizo Y, Nakanishi T. Tumor necrosis factor alpha (TNFα)-like factor produced by macrophages in rainbow trout, *Oncorhynchus mykiss.* Fish and Shellfish Immunology. 2001; 11:245

[91] Hirono I, Nam BH, Kurobe T, Aoki T. Molecular cloning, characterization, and expression of TNF cDNA and gene fron Japanese flounder *Paralychthys olivaceus.* Journal of Immunology. 2000; 165:4423

[92] Zou J, Wang T, Hirono I, Aoki T, Inagawa H, Honda T, Soma GI, Ototake M, Nakanishi T, Ellis AE, Secombes CJ. Differential expression of two tumor necrosis factor genes in rainbow trout, *Oncorhynchus mykiss.* Developmental and Comparative Immunology. 2002; 26:161

[93] Savan R, Sakai M. Presence of multiple isoforms of TNF alpha in carp (*Cyprinus carpio* L.): genomic and expression analysis. Fish and Shellfish Immunology. 2004; 17:87

[94] Zou J, Secombes CJ, Long S, Miller N, Clem LW, Chinchar VG. Molecular identification and expression analysis of tumor necrosis factor in channel catfish (*Ictalurus punctatus*). Developmental and Comparative Immunology. 2003; 27:845

[95] Garcia-Castillo J, Chaves-Pozo E, Olivares P, Pelegrin P, Meseguer J, Mulero V. The tumor necrosis factor α of the bony fish seabream exhibits the *in vivo* proinflammatory and proliferative activities of its mammalian counterparts, yet it functions in a species-specific manner. Cellular and Molecular Life Sciences. 2004; 61:1331

[96] Garcia-Castillo J, Pelegrin P, Mulero V, Meseguer J. Molecular cloning and expression analysis of tumor necrosis factor α from a marine fish reveal its constitutive expression and ubiquitous nature. Immunogenetics. 2002; 54:200

[97] Ordas MC, Costa MM, Roca FJ, Lopez-Castejon G, Mulero V, Meseguer J, Figueras A, Novoa B. Turbot TNFα gene: Molecular characterization and biological activity of the recombinant protein. Molecular Immunology. 2006; in press

[98] Zou J, Peddie S, Scapigliati G, Zhang, Bols NC, Ellis AE, Secombes CJ. Functional characterization of the recombinant tumor necrosis factors in rainbow trout, *Oncorhynchus mykiss.* Developmental and Comparative Immunology 2003; 27:813

[99] MacKenzie S, Planas JV, Goetz FW. LPS-stimulated expression of a tumor necrosis factor-alpha mRNA in primary trout monocytes and in vitro differentiated macrophages. Developmental and Comparative Immunology. 2003; 27:393

[100] Lindenstrom T, Secombes CJ, Buchmann K. Expression of immune response genes in rainbow trout skin induced by *Gyrodactylus derjavini* infections. Veterinary Immunology and Immunopathology. 2004; 97:137

[101] Yazawa R, Hirono I, Ohira T, Aoki T. Functional analysis of tumor necrosis factor gene promoter from Japanese flounder, *Paralichthys olivaceus*, using fish cell lines. Developmental and Comparative Immunology. 2005; 29:73

[102] Savan R, Kono T, Igawa D, Sakai M. A novel tumor necrosis factor (TNF) gene present in tandem with the TNF-α gene on the same chromosome in teleosts. Immunogenetics. 2005; 57:140

[103] Kono T, Zou J, Bird S, Savan R, Sakai M, Secombes CJ. Identification and expression analysis of lymphotoxin-beta like homologues in rainbow trout *Oncorhynchus mykiss*. Molecular Immunology. 2006; 43:1390

[104] Park CI, Kurobe T, Hirono I, Aoki T. Cloning and characterization of cDNAs for distinct tumor necrosis factor receptor superfamily genes from Japanese flounder *Paralichthys olivaceus*. Developmental and Comparative Immunology. 2003; 27:365

[105] Smith DE, Renshaw BR, Ketchem RR, Kubin M, Garka KE, Sims JE. Four new members expand the interleukin-1 superfamily. Journal of Biological Chemistry. 2000; 275:1169

[106] Verburg-van Kemenade BML, Weytes FAA, Debets R, Flik G. Carp macrphages and neutrophilic granulocytes secrete an interleukin-1-like factor. Developmental and Comparative Immunology. 1995; 19:59

[107] Yin Z, Kwang J. Carp interleukin-1β in the role of an immuno-adjuvant. Fish and Shellfish Immunology. 2000; 10:375

[108] Hong S, Zou J, Crampe M, Peddie S, Scapigliati G, Bols N, Cunningham C, Secombes CJ. The production and biodiversity of rainbow trout (*Oncorhynchus mykiss*) recombinant IL-1β. Veterinary Immunology and Immunopathology. 2001; 81:1

[109] Scapigliati G, Buonocore F, Bird S, Zou J, Pelegrin P, Falasca C, Prugnoli D, Secombes CJ. Phylogeny of cytokines: molecule cloning and expression analysis of sea bass *Dicentrarchus labrax* interleukin-1β. Fish and Shellfish Immunology. 2001; 11:711

[110] Wang Y, Wang Q, Baoprasertkul P, Peatman E, Liu Z. Genomic organization, gene duplication, and expression analysis of interleukin-1β in channel catfish (*Ictalurus punctatus*). Molecular Immunology. 2006; 43:1653

[111] Engelsman MY, Stet RJM, Schipper H, Verburg-van Kemenade BML. Regulation of interleukin 1 beta RNA expression in the common carp, *Cyprinus carpio* L. Developmental and Comparative Immunology. 2001; 25:195

[112] Mathew JA, Guo YX, Goh KP, Chan J, Verburg-van Kemenade BML, Kwang J. Characterization of a monoclonal antibody to carp IL-1β and the development of a sensitive capture ELISA. Fish and Shellfish Immunology. 2002; 13:85

[113] Hong S, Peddie S, Campos-Perez JJ, Zou J, Secombes CJ. The effect of intraperitoneally administered recombinant IL-1β on immune parameters ad resistance to *Aeromonas salmonicida* in the rainbow trout (*Oncorhynchus mykiss*). Developmental and Comparative Immunology. 2003; 27:801

[114] Hong S, Zou Jun, Collet B, Bols NC, Secombes CJ. Analysis and characterization of IL-1β processing in rainbow trout, *Oncorhynchus mykissi.* Fish and Shellfish Immunology. 2004; 16:453

[115] Peddie S, Zou J, Cunningham C, Secombes CJ. Rainbow trout (*oncorhynchus mykiss*) recombinant IL-1β and derived peptide induce migration of head-kidney leucocytes *in vitro*. Fish and Shellfish Immunology. 2001; 11:697

[116] Peddie S, Zou J, Secombes CJ. A biologically active IL-1B derived peptde stimulates pahagocytosis and bacterial activity in rainbow trout *Oncorhynchus mykiss* (Walbaum), head kidney leukocytes *in vitro*. Journal of Fish Diseases. 2002; 25:351

[117] Sangrador-Vegas A, Martin SAM, O'Dea PG, Smith TJ. Cloning and characterization of the rainbow trout (*Oncorhynchus mykiss*) type II interleukin-1 receptor cDNA. European Journal of Biochemistry. 2000; 267:7031

[118] Subramanian S, Stansberg C, Olsen L, Zou J, Secombes CJ, Cunningham C. Cloning of a *Salmo salar* interleukin-1 receptor-like cDNA. Developmental and Comparative Immunology. 2002; 26:415

[119] Robertsen B. The interferon system of teleost fish. Fish and Shellfish Immunology. 2006; 20:172

[120] Altmann SM, Mellon MT, Distel DL, Kim CH. Molecular and functional analysis of an interferon gene from the zebrafish, *Danio rerio*. Journal of Virology. 2003; 77:1992

[121] Bergan V, Rokenes T, Larsen R, Albuquerque A. Atlantic salmon interferon genes: cloning, sequence analysis, expression, and biological activity. Journal of interferon and cytokine research. 2003; 23:601

[122] Long S, Wison M, Bengten E, Bryan L, Clem LW, Miller NW, Chinchar VG. Identification of a cDNA encoding channel catfish interferon. Developmental and Comparative Immunology. 2004; 28:97

[123] Robertson B, Zou J, Secombes C, Leong JA. Molecular and expression analysis of an interferon-gamma-inducible guanylate-binding protein from rainbow trout (*Oncorhynchus mykiss*). Developmental and Comparative Immunology. 2006; 30:1023

[124] Shen L, Stuge TB, Bengten E, Wilson M, Chinchar VG, Naftel JP, Bernanke JM, Clem LW, Miller NW. Identification and characterization of clonal NK-like cells from channel catfish (*Ictalurus punctatus*). Developmental and Comparative Immunology. 2004; 28:139

[125] Zou Jun, Bird S, Truckle J, Bols N, Horne M, Secombes CJ. Identification and expression analysis of an IL-18 homologue and its alternatively spliced from in rainbow trout (*Oncorhynchus mykiss*). European Journal of Biochemistry. 2004; 271:1913

[126] Yoshiura Y, Kiryu I, Fujiwara A, Suetake H, Suzuki Y, Nakanishi T, Ototake M. Identification and characterization of Fugu orthologues of mammalian interleukin-12 subunits. Immunogenetics. 2003; 55:296

[127] Platanias LC, Fish EN. Signaling pathways activated by interferons. Experimental Hematology. 1999; 27:1583

[128] Lutfalla G, Crollius HR, Stange-thomann N, Jaillon O, Mogensen K, Monneron D. Comparative genomic analysis revels independent expansion of a lineage-specific gene family in vertebrates: The class II cytokine receptors and their ligands in mammals and fish. BMC Genomics. 2003; 4:29

[129] Herpin A, Lelong C, Favrel P. Transforming growth factor-β-related proteins: an ancestral and widespread superfamily of cytokines in metazoans. Developmental and Comparative Immunology. 20004; 28:461

[130] Stafford J, Neumann NF, Belosevic M. Inhibition of macrophage activity by mitogen-induced goldfish leukocyte deactivating factor. Developmental and Comparative Immunology. 1999; 23:585

[131] Jang SI, Hardie LJ, Secombes CJ. Effects of transforming growthfactor β1 on rainbow trout *Oncorhynchus mykiss* macrophage respiratory burst activity. Developmental and Comparative Immunology. 1994; 18:315

[132] Hardie LJ, Laing KJ, Daniels GD, Grabowski PS, Cunningham C, Secombes CJ. Isolation of the first piscine transforming growth factor β gene: analysis reveals tissue specific expression and a potential regulatory sequence in rainbow trout (*Oncorhynchus mykiss*). Cytokine. 1998; 10:555

[133] Sumathy K, Desai KV, Kondaiah P. Isolation of transforming growth factor-β2 cDNA from a fish, *Cyprinus carpio* by RT-PCR. Gene. 1997; 191:103

[134] Tafalla C, Aranguren R, Secombes CJ, Castrillo JL, Novoa B, Figueras A. Molecular characterization of sea bream (*Sparus aurata*) transforming growth factor β1. Fish and Shellfish Immunology. 2003; 14:405

[135] Barreda DR, Hanington PC, Walsh CK, Wong P, Belosevic M. Differentially expressed genes that encode potential markers of goldfish macrophage development in vitro. Developmental and Comparative Immunology. 2004; 28:727

[136] Bienek DR, Belosevic M. Comparative assessment of growth of *Trypanosoma danilewskyi* (Laveran & Mesnil) in medium containing fish or mammalian serum. Journal of Fish Disease. 1997; 20:217

[137] Pfeffer K, Biological functions of tumor necrosis factor cytokines and their receptors. Cytokine and Growth Factor Reviews. 2003; 14:185

[138] Neumann NF, Stafford JL, Belosevic M. Biochemical and functional characterization of macrophage stimulating factors secreted by mitogen-induced goldfish kidney leucocytes. Fish and Shellfish Immunology. 2000; 10:167

[139] Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nature Reviews Immunology. 2005; 5:953

[140] Belosevic M, Hanington PC, Barreda DR. Development of goldfish macrophages *in vitro*. Fish and Shellfish Immunology. 2006; 20:152

[141] Weigmann A, Corbeil D, Hellwig A, Huttner WB. Prominin, a novel microvilli-specific polytopic membrane protein of the apical surface of epithelial cells, is targeted to plasmalemmal protrusions of non-epithelial cells. Proceeding of the National Academe of Science. 1997; 94:12425

[142] Corbeil D, Roper K, Hellwig A, Tavian M, Miraglia S, Watt SM, Simmons PJ, Peault B, Buck DW, Huttner WB. The Human AC133 Hematopoietic Stem Cell Antigen Is also Expressed in Epithelial Cells and Targeted to Plasma Membrane Protrusions. Journal of Biological Chemistry. 2000; 275:5512

[143] Fargeas CA, Florek M, Huttner WB, Corbeil D. Characterization of Prominin-2, a New Member of the Prominin Family of Pentaspan Membrane Glycoproteins. Journal of Biological Chemistry. 2003; 278:858

[144] Miraglia S, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, Bray Ra, Waller EK, Buck DW. A Novel Five-Transmembrane Hematopoietic Stem Cell Antigen: Isolation, Characterization, and Molecular Cloning. Blood. 1997; 90:5013

[145] Yu Y, Flint A, Dvorin EL, Bischoff J. AC133-2 a Novel Isoform of Human AC133 Stem Cell Antigen. Journal of Biological Chemistry. 2002; 277:20711

[146] Shmelkov SV, Jun L, St Clair R, McGarrigle D, Derderian CA, Usenko JK, Costa C, Zhang F, Guo X, Rafii S. Alternative promoters regulate transcription of the gene that encodes stem cell surface protein AC133. Blood. 2004; 103:2055

[147] Maw MA, Corbeil D, Koch J, Hellwig A, Wilson-Wheeler JC, Bridges RJ, Kumaramanickavel G, John S, Nancarrow D, Roper K, Weigmann A, Huttner WB, Denton MJ. A frameshift mutation in prominin (mouse)-like 1 causes human retinal degeneration. Human Molecular Genetics. 2000; 9:27

[148] Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MAS, Rafii S. Expression of VEGFR-2 and AC133 by circulating human CD34$^+$ cells identifies a population of functional endothelial precursors. Blood 2000; 95:952

[149] Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, Tsukamoto AS, Gage FH, Weissman IL. Direct isolation of human central nervous system stem cells. Proceedings of the National Academe of Science. 2000; 97:12425

[150] Richardson GD, Robson CN, Lang SH, Neal DE, Maitland NJ, Collins AT. CD133, a novel marker for human prostatic epithelial stem cells. Journal of Cell Science. 2004; 117:3539

[151] Roper K, Corbeil D, Huttner WB. Retention of prominin in the mcrovilli reveals distinct cholesterol-based lipid microdomains in the apical plasma membrane. Nature Cell Biology. 2000; 2:582

[152] Corbeil D, Roper K, Fargeas CA, Joester A, Huttner WB. Prominin: A Story of Cholesterol, Plasma Membrane Protrusions and Human Pathology. Traffic. 2001; 2:82

[153] Marzesco AM, Janich P, Wilsch-Brauninger M, Dubreuil V, Langenfeld K, Corbeil D, Huttner WB. Release of extracellular membrane particles carrying the stem cell marker prominin-1 (CD133) from neural progenitors and other epithelial cells. Journal of Cell Science. 2005; 118:2849

[154] Corbeil D, Roper K, Fargeas CA, Joester A, Huttner WB. Prominin: A Story of Cholesterol, Plasma Membrane Protrusions and Human Pathology. Traffic. 2001; 2:82

[155] Fang FC. Antimicrobial reactive oxygen species and nitrogen species: concepts and controversies. Nature Reviews Immunology. 2004; 2:820

[156] Levy O. Antimicrobial proteins and peptides: anti-infective molecules of mammalian leukocytes. Journal of Leukocyte Biology. 2004; 76:909

[157] Stenger S. Immunological control of tuberculosis: role of tumor necrosis facto and more. Annals of Rheumenological Disease. 2005; 64:24

[158] Hume DA. The mononuclear phagocytic system. Current Opinion in Immunology. 2006; 18:49

[159] Roach DR, Bean AGD, Demangel C, France MP, Briscoe H, Britton WJ. TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of Mycobacterial infection. Journal of Immunology. 2002; 168:4620

[160] Rothlein R, Czajkowski M, O'Neill MM, Marlin SD, Mainolfi E, Merluzzi VJ. Induction of intercellular adhesion molecule 1 on primary and continuous cell lines by pro-inflammatory cytokines. Regulation by pharmacologic agents and neutralizing antibodies. Journal of Immunology. 1988; 141:1665

[161] Roach DR, Bean AG, Demangel C, France MP, Briscoe H, Britton WJ. TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. Journal of Immunology. 2002; 168:4620

[162] Appelburg R. Macrophage nutriprive antimicrobial mechanisms. Journal of Leukocyte Biology. 2006; 79:1117

[163] Cadwallader KA, Condliffe AM, McGregor A, Walker TR, White JF, Stephens LR, Chilvers ER. Regulation of phosphatidylinositol 3-kinase activity and phosphatidylinositol 3,4,5-trisphosphate accumulation by neutrophil priming agents. Journal of Immunology. 2002; 169:3336

[164] Mehta M, Woo PT. Acquired cell-mediated protection in rainbow trout, Oncorhynchus mykiss, against the haemoflagellate, Cryptobia salmositica. Parasitology Research. 2002; 88:956

[165] Chaves-Pozo E, Munoz P, Lopez-Munoz A, Pelegrin P, Garcia Ayala A, Mulero V, Meseguer J. Early innate immune response and redistribution of inflammatory cells in the bony fish gilthead seabream experimentally infected with Vibrio anguillarum. Cell Tissue Research. 2005; 320:61

[166] Traver D, Paw BH, Poss KD, Penberthy WT, Lin S, Zon LI. Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. Nature Immunology. 2003; 4:1238

[167] Barreda DR, Hanington PC, Stafford JL, Belosevic M. A novel soluble form of the CSF-1 receptor inhibits proliferation of self-renewing macrophages of goldfish (Carassius auratus L.). Developmental and Comparative Immunology. 2005; 29:879

[168] Hanington PC, Barreda DR, Belosevic M. A novel hematopoietic granulin induces proliferation of goldfish (Carassius auratus L.) macrophages. Journal of Biological Chemistry. 2006; 281:9963

[167] Barreda DR, Hanington PC, Stafford JL, Belosevic M. A novel soluble form of the CSF-1 receptor inhibits proliferation of self-renewing macrophages of goldfish (Carassius auratus L.). Developmental and Comparative Immunology. 2005; 29:879

[168] Hanington PC, Barreda DR, Belosevic M. A novel hematopoietic granulin induces proliferation of goldfish (Carassius auratus L.) macrophages. Journal of Biological Chemistry. 2006; 281:9963

APPENDIX

Goldfish TNFα-2 partial sequence containing complete open reading frame. The UTR is highlighted.

```
  1 ggacatagct gaacttacta acagacacaa tgatgataga tgttgagagt cagttagctg
 61 aagaaggagc gcaggtgacg gtgtcgagga ggaggtccgg tgtctggcgg gtgtgtgggg
121 tcctgctggc tgtggccctg tgtgccgccg ctgctgtctg cttcacattc aacaagtctc
181 agaacaatca ggaaagtgga aatgagctga ggctcacatt aagagaccat ctttcaaaag
241 aaaatgtcac ttccaaggcc gcgatccatt taacaggtgc atatgaccct gacgtgtgta
301 cggacaacct agactggaaa cagaaccagg accaggcttt tgtttcaggt ggcttgaaac
361 tagtggacag agagatcatc attccttacg acggcattta cttcgtctac agtcaggtgt
421 ctttccacat cagctgcaag gctgacgtga ctgaggaaca cgagggcgtg cacatgagcc
481 acgcagtgtt gcgcttctcg gagtcctacg ccagctacaa gccgctcttc agcgcgatcc
541 gctcggcctg cgtgcacgcg actgacactg aagacctgtg gtacaacacc atttacctcg
601 gagcggcctt caacctgcga gctggagaca aactgcgcac cgacaccacc acagaactcc
661 tgccccgcgt ggaaagcgaa aacggaaaga ccttcttcgg ggtgtttgct ttatga
```

Goldfish prominin partial sequence containing complete open reading frame. The UTR is highlighted

```
   1 aagcagtggt atcaacgcag agtacgcggg gactcaatgc agctaaactt cagtgttgac
  61 atctgggata aagacatcag atcttcagac tcatttgagc tgaactaaaa gaggaactcg
 121 ctcaacaata gctggagaac tttaaaacag gtgacatctg atgaccgtaa gaatcgagaa
 181 atgaccttaa aacctactgg ctaatgctca acatgaggtc cagaatgaaa aaggtttttt
 241 ggagtggctt tttgctcctg tttctctgga cattggtaaa tggacaagtg tcctgtcctg
 301 tagagacgac gagaacagcg ctccaaactc cagatgaaat accaaccaag ctgaccttca
 361 acagtgcctt tatgtctcca atggtacatt cattcctggg ctctgtccag tccaatccat
 421 tccctaaaga tatactcatt ggaatcacaa aaggttacga cacagagacg gttaaagagg
 481 ttttgcaata ccagacagga ttcttggtct gcgtggccat tgggatcctg tacattgtcc
 541 tgatgcctct aattggcctg ttctttgcct gctgtcgttg ctgtggtaac tgcgggggcc
 601 gcatgcagca gaagaaaaca acaaacatcc actgcaagcg aagaagcttt tacttgtgca
 661 catttctcat cactgtacta attatggcag ggaacatctg cgtgttcctg agcagtacaa
 721 acacttcaga gactttgatg cgcagcccca cagaactcac cgacatcttg gagaatgtca
 781 aaggctatct taactccatt cctcagcaaa tccaacaagt gacgaatgag agccgtgtga
 841 ctgtggatac ggtcaaaaac aacttagttg aaacagggcc tttgcttggc aaactgatcc
 901 agaatggtct taagggccac cttgacccag ccttaaactc cactactgaa ataggccaag
 961 tgataaacag caccagcgat ggactgctgc agcttaataa aaccttggga gctgctgaaa
1021 ccgaaggcgg ccgcgctgca agctatttat ctgcggtgag gcagaggatt aacaacactt
1081 tgcgtatgtc agactgtgta aactgcgctg cccttcagcc agaactggat aaactatcac
1141 tggatggaag ccttgaattt cctgatcaaa aggacctgag gtctgctgta gacaaggcta
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1261 gaaaggtgaa aaatgagacc agactaagtg tgcagggggc tctattggag cttgaaaatt
1321 taaaaacccg gatcttaagt gtgactagag accttcctct taatgtgttc aacactacct
1381 cagcatcact aacggacatg cagaaatcga tcaaaggttt tgctccttat atagagcgat
1441 caagccaaat cagccgggat gttggattgg tcatgagctg tctgattttg ctggtggtga
1501 tctgtaactt tctgggtctg ctgctgggca tagctggact aaatcctaaa gacaatccct
1561 ctgaacgttc tggcacatct aactgtggag gaatattttt catggcagga gtaggtttca
1621 gcttcctggt ctcctggatc ttcatgttag tagtgttgat acttttcata gtgggaggaa
1681 atacctatac cctgatctgt aaagccatgg caaaccaagg aactcatcca gcttatgaca
1741 ctccagggct gattccaggt ctgaacctgt ccacggtcct gaatttgaaa acaaacctga
1801 atattataaa tgtgtacagt gactgtcaga agaatatgcc gttgtggacc acatttcatc
1861 tgaatgagat cttcgatcta aatagtaaat tagatgtttc acagtacact aatgagattg
1921 atcagacctt taatgggtta caaatcaaca tagcaaacat caccatcctc agtccagagg
1981 taaagtccca gctaaacaac ttctccagca ggaccagcag tatgaacttc agcaacatta
2041 tacatcagat taatgacttg tcagggaatg atctgagctc agtagcagaa agtctggatg
2101 tcttggctgg caaacagagt aaccaatcta aaaaagatga actgcacggg gaagcaaaag
2161 atctgcgtga catccagact gacgtcatgt ctaacattat gcctttgctg aaggaactga
2221 acagcactgt taagaatctc agtgaagaag catcacaaat cactgcagct atggaaaatg
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2281 ttttcaaaga agttggctat gctcaggacg tcctgaacta caacatttca aaaatagtgc
2341 aaactgaaag cagggcattc gtagactgtc agataaaaat ctttcagaca tttctttact
2401 gggcaaatca aacgatcaca gagaaggtgg gtcgttgtgg gcctgcagct gcagcagtag
2461 acagatcaga ggagctcgtc tgtaaacact tggtagaatc tttgaatgcc ttttggttga
2521 gtttgggctg gtgtatgatg ttcctgatcc ccagcatcat tttctcagtt aaactggcca
2581 agtactaccg caggatgaag tattcagatg cttatgagaa caataacttc atgatgaatc
2641 ctttcccaaa ggccacctca aacctgaatt attgggaaat ggcaaacaac cacattttac
2701 ataagcacat aaattaaaat gacggttaag ttattaaaat tgacccacag atcaat